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Bulbar Influence on Spinal Cord Dorsum Potentials and Ventral Root Reflexes.

By

U. F. Lindblom and J. O. Ottosson

Received 8 August 1955.

Introduction.

Influence from the brain stem on spinal reflex activity was first demonstrated by SETSCHENOV (1863) in experiments on frogs and more recent electrophysiological studies on cats have further emphasized the significance of the brain stem control of spinal motor activity. Several authors have shown that electrical stimulation of the reticular formation may result in both inhibition and facilitation of reflexly or cortically induced cord activity recorded myographically or from the ventral roots (see *e.g.* LLOYD 1941, MAGOUN and RHINES 1946, RHINES and MAGOUN 1946, HODES, PEACOCK and HEATH 1951, ELDRED, GRANIT and MERTON 1953, GERNANDT and THULIN 1955).

The influence from higher centers upon *afferent* transmission at spinal levels has been far less studied. In an investigation into the suprabulbar influence on spinal reflexes AUSTIN (1952) observed that inhibition of polysynaptic reflexes was accompanied by a decrease of the negative intermediary cord dorsum potential evoked by a dorsal root volley. As the negative intermediary potential signals internuncial activity (GASSER and GRAHAM 1933) AUSTIN suggested that the decrease was due to descending inhibitory influence on spinal interneuron pools.

In an investigation of the cord dorsum potentials elicited from different kinds of peripheral nerves BERNHARD (1952, 1953) found that stimulation

of low threshold cutaneous fibres evokes a cord dorsum negativity (the N_1 deflection) which is a main fraction of the negative intermediary potential of GASSER and GRAHAM. The N_1 deflection is derived from the dorsal horn around the root entry zone and is mainly of postsynaptic origin (BERNHARD 1952, 1953, BERNHARD, LINDBLOM and OTTOSSON 1953, LINDBLOM and OTTOSSON 1953 a, AUSTIN and MCCOUCH 1954). It probably signals transmission to suprasegmental levels (BERNHARD and WIDÉN 1953, BOHM 1953).

In a previous paper (LINDBLOM and OTTOSSON 1953 b) it was reported that spinal section can be followed by an augmentation of the N_1 deflection in decerebrate cats. This observation indicates the existence of a descending suppressor system originating in the brain stem and displaying a tonic inhibitory influence on the N_1 generating neurons. The result was taken as an evidence of brain stem influence on afferent transmission at spinal levels. The investigation was pursued by experiments showing that the N_1 deflection could be depressed by electrical stimulation at some points in the bulbar reticular formation as reported in a preliminary communication (LINDBLOM and OTTOSSON 1954). HAGBARTH and KERR (1954) found that the relayed dorsal and ventral column responses evoked from a dorsal root could be depressed by electrical stimulation of various central structures, among others the reticular formation. They concluded that synaptic afferent transmission in the spinal cord can be influenced by descending pathways. HAGBARTH and KERR also noted that the negative intermediary cord dorsum potential decreased in parallel with the relayed dorsal column response and the dorsal root reflex (see also KLEYNTJENS, KOIZUMI and BROOKS 1954, 1955).

A full account will be given in this paper of the effect of electrical stimulation of the bulbar reticular formation on the spinal cord dorsum potentials, elicited from skin and muscle afferents. The results are discussed in relation to descending influence on spinal afferent transmission. A comparison is made with the effect on the ventral root reflex discharges. Attempts have been made to avoid widespread excitation of the conditioning bulbar stimulus. Due precautions have been taken to prevent effects from antidromic activation of ascending spinal fibre systems.

Methods.

Cats narcotized with Nembutal (20—40 mg./kg. intravenously) were used. Small doses of d-tubocurarine were given repeatedly to prevent all movements when the brain stem and the peripheral nerves were stimulated.

The blood loss at the operation was compensated for by intravenous infusion of a plasma substitute solution (Macrodex 6 % Pharmacia). A 5 per cent glucose solution was given continuously by an intravenous drip.

The L_7 and S_1 ventral roots as well as the sural nerve, the pad nerve and the two nerves to the gastrocnemius muscle were prepared and cut distally. The bulb was exposed by resection of the occipital bone and the cerebellum. All exposed nervous tissues were covered by liquid paraffin which was kept at 38° C.

The cord dorsum potentials were recorded with one electrode in the midline on the cord dorsum and the other in non-neural tissue. The head of the cat was fixed in a modified Horsley-Clarke apparatus and needle electrodes were inserted by means of micrometer gauges to different depths in the bulb. The needles were covered with lacquer except for the tip (0.1—0.2 mm.). The lacquer insulation was checked both microscopically and by resistance measurements. An indifferent electrode was inserted in the neck muscles. The specificity of the responses obtained from adjacent stimulation points in the bulb indicated that the excitation probably did not exceed 0.5 mm. with the voltages used (*cf. e.g.* LILJESTRAND 1953). Each bulbar point (0.5 mm. apart) was tested for electrical response from ascending fibres activated from skin and muscle nerves. Each point was also stimulated with a single impulse and records were taken from the lumbar cord surface and the ventral roots. For bulbar stimulation square wave pulses of 0.4—2 msec. duration were used singly or repetitively at a frequency of 150 per second. The stimulus strength was usually around 5 volts, higher voltages than 12 volts were avoided. The peripheral nerves were stimulated with single pulses of 0.1 msec. duration. A condensor coupled differential amplifier with a time constant of 30 msec. was used.

After the experiment the inserted needles were left in the bulb and the preparation was fixed *in situ* in 10 per cent formol for a quarter of an hour before fixation in the ordinary way. The needle tracks were easy to follow on serial frontal sections. The following results are based upon observations from 30 needle tracks in 15 cats. The needle tracks were evenly distributed to 5 different levels (I—V in Fig. 1) in the lower brain stem and were located both laterally and medially. A complete mapping was not attempted.

Results.

Description of the Conditioning Response.

Fig. 2 A shows the lumbar cord dorsum response to a single shock delivered to the reticular formation in the brain stem. It consists of two spike potentials separated by an interval of about 1 msec. followed by a slow positive deflection. The first spike has a latency corresponding to a conduction velocity of about 100 m. per second. When stimulation was repetitive the spike potentials followed high frequencies whereas the slow positive deflection decreased in amplitude. In

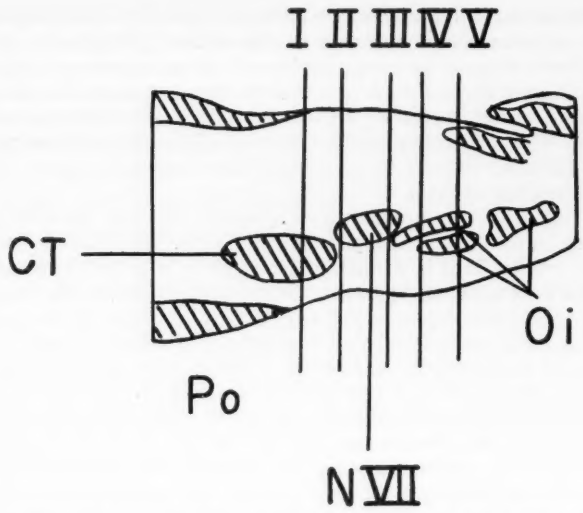

Fig. 1. Midsagittal reconstruction of the lower brain stem. The levels explored by stimulation are designated by Roman figures. Abbreviations: CT, corpus trapezoides; N VII, nucleus nervi facialis; Oi, nucleus olivaris inferior; Po, pons.

the present investigation the response in Fig. 2 A was taken as an index of activity in reticulospinal pathways and segmental interneurons.

Conditioning Experiments.

Since a single conditioning shock in the bulb proved to be ineffective repetitive stimulation was used. The shortest time for conditioning stimulation which evoked significant effects was found to be 50 msec. at a repetitive rate of 150 per second. It means that the conditioning stimulus consisted of a train of 7 impulses. As the conditioning stimulus evoked a disturbance of the base line, the test responses were elicited a few msec. after the last conditioning shock. Control records showed that the results were qualitatively the same when the test responses were elicited during the conditioning stimulation. The test stimuli were applied to peripheral nerves on the same side as the needle electrode inserted in the brain stem. Contralateral effects were not systematically studied.

Effect on N_1 deflection. Fig. 2 B illustrates the cord dorsum response

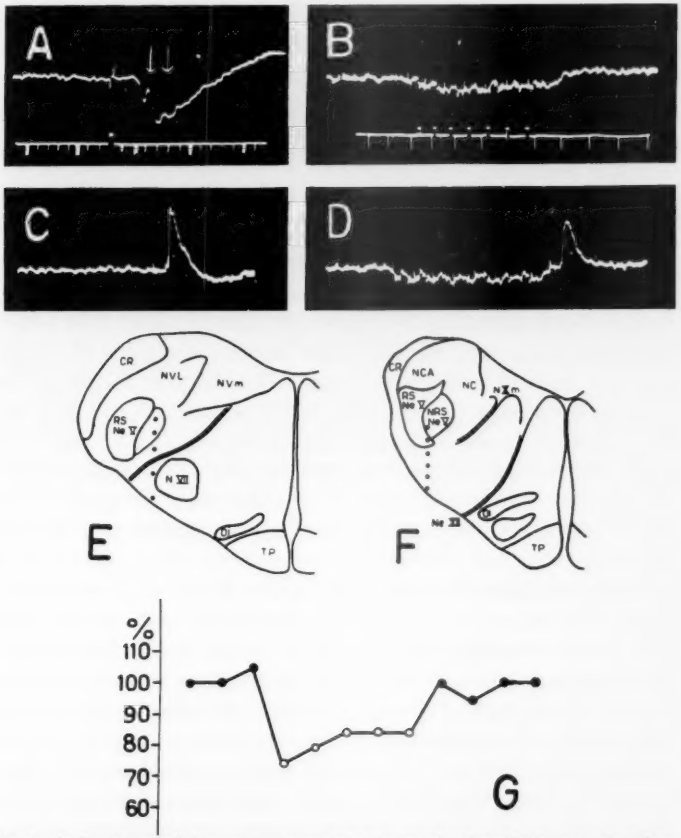

Fig. 2. A: lumbar cord dorsum response to a single stimulus of 0,4 msec. duration in the bulbar reticular formation. Arrows indicate the two spike potentials which precede the slow positive deflection. Time in msec. and 5 msec. B: cord dorsum response elicited by a train of 7 volleys of 2 msec. duration at 150 per sec. and lower stimulus strength than in A. Time in B, C and D in 10 msec. C: unconditioned lumbar cord dorsum response (N_1 deflection) to low threshold stimulation of the sural nerve. D: same preceded by a conditioning response as in B. E (level III) and F (level V) show points in which stimulation caused a reduction of the N_1 deflection. G: amplitude values of the N_1 deflection on consecutive sweeps (interval 2 sec.). Filled circles represent unconditioned responses and open circles conditioned responses. The average unconditioned value is taken as 100 per cent.

Abbreviations: (see also Fig. 1) CR, corpus restiforme; NC, nucleus fasciculi cuneati (Burdach;) NCA, nucleus accessorius fasciculi cuneati; NRSNeV, nucleus radicis spinalis nervi trigemini; NVL, nucleus lateralis nervi vestibuli (Deiters); NVm, nucleus medialis nervi vestibuli (Bechterew); NXm, nucleus originis nervi vagi; N XII, nucleus nervi hypoglossi; Ne XII, nervus hypoglossus; TP, tractus pyramidalis.

to a short train of conditioning stimuli, the stimulus strength being much lower and the sweep slower than in A so details are difficult to recognize. Record C shows the N_1 deflection elicited by stimulation of the sural nerve without, record D with preceding conditioning stimulation. As seen the N_1 deflection following the conditioning stimuli is depressed.

A significant depression of the N_1 deflection was obtained from certain points along 4 out of 30 needle tracks. The sketches in Fig. 2 E and F illustrate two of the four needle tracks. Black circles indicate the points the stimulation of which depressed the N_1 deflection elicited from the sural nerve. Fig. 2 G shows the effect obtained from one of the points in the sketches. The circles represent the value of the test response from 12 consecutive sweeps. The mean amplitude of the unconditioned response was taken as 100 per cent. Open circles are the amplitude values of the conditioned responses all of which fall below the unconditioned values (filled circles). The average decrease in amplitude was 20 per cent which agrees with the result of the other experiments. A facilitation of the N_1 deflection was never observed.

The cord dorsum response of the type in Fig. 2 A was of uniform appearance though it was elicited from various parts in the bulb. There were, however, three important exceptions: (i) When points within the bulbar projection area of the ascending anterolateral fibre system were stimulated, a more complex cord dorsum response than that in Fig. 2 A was obtained. Besides, recording from the previously stimulated points in this area showed a response following peripheral nerve stimulation. When such points were chosen for conditioning stimulation a reduction of the N_1 deflection could easily be obtained. It was, however, impossible to exclude that the reduction was due to antidromic activation of ascending axons from N_1 generating neurons. Therefore, only those points are considered in the present investigation whose stimulation evoked a cord dorsum response of the simple type shown in Fig. 2 A. (ii) The points which were located within the projections of the dorsal column fibre system were easily recognized as stimulation at these points evoked a typical cord dorsum negativity due to antidromic activation of N_1 generating neurons, as well as back-firing in peripheral nerves. (iii) Stimulation in the pyramids evoked a characteristic cord dorsum response the influence of which has not been considered in this investigation.

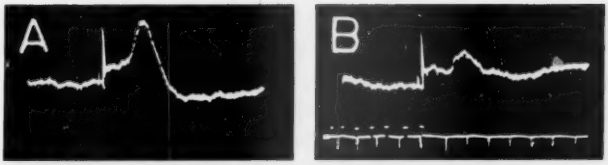

Fig. 3. Lumbar cord dorsum response to stimulation of muscle afferents in the gastrocnemius nerve. Record A shows the unconditioned response which consists of an afferent spike and a small negativity evoked from low threshold fibres and a larger late negativity elicited from high threshold fibres. In record B the response is conditioned by a train of 7 impulses of 2 msec. duration at 150 per second delivered to a point in the bulboreticular formation ipsilateral to the nerve stimulated. The late negativity is depressed while the other components are uninfluenced. Time in 10 msec.

The ventral root reflexes were also recorded when a depression of the N_1 deflection was obtained. The polysynaptic reflex elicited from the same cutaneous nerve as the N_1 deflection was usually depressed. From certain points it was, however, unaffected and it could even be augmented. The monosynaptic reflex elicited from the ipsilateral gastrocnemius nerve was always increased.

From most bulbar points investigated no reduction of the N_1 deflection could be evoked though the conditioning stimulus influenced various other spinal cord activities.

Effect on cord dorsum response to muscle nerve stimulation. The cord dorsum response elicited from muscle afferents has been described by BERNHARD (1952, 1953). When low threshold fibres are excited the response consists of a primary afferent spike and a small negativity following immediately upon the spike. None of these responses was ever influenced by stimulation in the reticular formation. (Fig. 3). The cord dorsum response to stimulation of high threshold muscle afferents consists of a slow negative deflection of rather high amplitude and with a latency of about 10 msec. Fig. 3 A illustrates the cord dorsum response to stimulation of muscle afferents. Record B shows the depressive effect on the late negative deflection of a train of conditioning reticular stimuli. The same effect was obtained from several points in different bulbar regions, some of which are illustrated in Fig. 4 by horizontal scores (isolated or across other symbols). The reduction of the amplitude of the late negative deflection

often amounted to 50 per cent and values as high as 75 per cent were sometimes obtained. An increase in the amplitude, as a consequence of conditioning bulbar stimulation, was never observed.

Effects on different reflexes. As mentioned the monosynaptic reflex was facilitated and the polysynaptic reflex usually inhibited when the bulbar points were stimulated from which the N_1 deflection was depressed. The reflexes were also tested after stimulation at points in the reticular formation with no influence on the N_1 deflection. For illustration of the results 5 needle tracks from three different experiments are reproduced in Fig. 4. The sketches A, B and C correspond to the levels II, IV, and V in Fig. 1. Conditioning stimulation at some of the points resulted in inhibition of both the monosynaptic and polysynaptic reflexes (filled circles) whereas stimulation at other points resulted in inhibition or facilitation of one of the reflexes, while the other was not influenced. Thus the monosynaptic reflex could be facilitated (open squares) or inhibited (open circles) when the polysynaptic reflex was unaffected, or the latter could be inhibited (open triangle) when the monosynaptic reflex was unchanged. Stimulation at still other points evoked facilitation of the monosynaptic reflex and inhibition of the polysynaptic reflex (filled triangles). Facilitation of the polysynaptic reflex was a comparatively uncommon observation. As illustrated in the figure the effect of the conditioning stimulation often changed when the stimulating electrode was moved 0.5 mm.

The reflex response elicited from high threshold muscle afferents was often depressed. The depression occurred whether the preceding monosynaptic reflex was inhibited or facilitated and was independent of the effect on the cord dorsum response evoked from high threshold muscle fibres. A significant facilitation of this reflex response was not observed.

Discussion.

The conditioning response used in the present investigation had a uniform appearance when the stimulus was applied to different points in the reticular formation. The response had the same characteristics as those found by LLOYD (1941) by means of intraspinal recording from the ventral horn after bulbar stimulation. LLOYD interpreted the spikes as signalling activity in reticulospinal and propriospinal fibres respectively, while the slow positive deflection was assumed to represent activity in local interneuron pools. It seems reasonable to

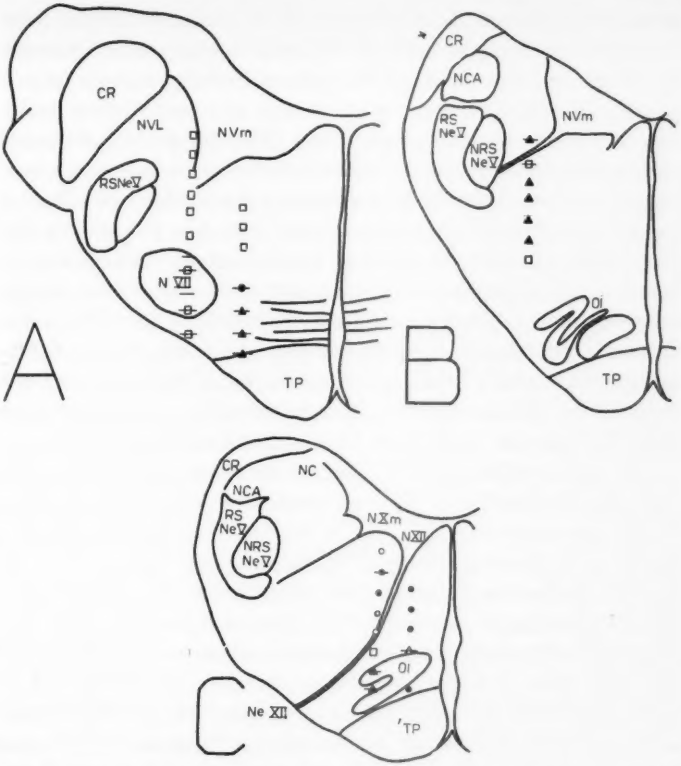

Fig. 4. Localization of bulbar points stimulated in conditioning experiments. Sections A, B, and C refer to levels II, IV, and V respectively in Fig. 1. Symbols represent the conditioning effect as follows:

● inhibition of the monosynaptic and the polysynaptic reflex
○ inhibition of the monosynaptic reflex
□ facilitation of the monosynaptic reflex
△ inhibition of the polysynaptic reflex
▲ inhibition of the polysynaptic reflex and facilitation of the monosynaptic reflex
— inhibition of the late cord dorsum response evoked from high threshold muscle afferents.

Abbreviations as in Fig. 2.

assume that the impulses which mediate the segmental effects in this investigation are transmitted via the bulbospinal correlation system of LLOYD.

In the bulbo-reticular formation sites were found where stimulation

caused a reduction of the amplitude of the N_1 deflection elicited from a cutaneous nerve in the hind leg. The result implies that internuncial activity in the dorsal horn can be inhibited from the reticular formation. As the N_1 deflection signals activity in a spino-bulbar transmission system (BERNHARD and WIDÉN 1953, BOHM 1953) the result further indicates a descending inhibitory influence on ascending spinal conduction from low threshold cutaneous fibres. Since no influence has been noticed on the afferent spike potentials (HAGBARTH and KERR 1954) it seems reasonable to assume that the bulbar influence is exerted on postprimary neurons although other possibilities are not excluded (see *e.g.* MCCULLOCH, LETTVIN, PITTS and DELL 1952). The present investigation is complementary to earlier experiments (LINDBLOM and OTTOSSON 1953 b) in which section of the spinal cord was followed by an increase of the N_1 deflection. The results are further consistent with the conclusions of HAGBARTH and KERR (1954).

The reticular sites with inhibitory influence on the N_1 deflection were few compared with the great number of points from where influence on motor activity could be elicited. This fact may imply a limited basis for reticulospinal influence on sensory systems. It is also remarkable that the reduction of the N_1 deflection was of small order, seldom exceeding 20 per cent. For comparison the cord dorsum negativity evoked from high threshold muscle afferents could be reduced by 75 per cent. The comparatively small degree of reduction of the N_1 deflection may be accounted for by the powerful synaptic articulations between the afferent fibres and the N_1 generating neurons (BERNHARD 1953). Another explanation may be based upon recent observations by AUSTIN and MCCOUCH (1954) implying that part of the N_1 deflection is presynaptic in origin.

The N_1 deflection could be reduced whether the polysynaptic reflex elicited from the same cutaneous nerve was reduced, unaffected or facilitated. This fact supports the conclusion by BERNHARD and WIDÉN (1953) that the N_1 deflection need not participate in the segmental reflex transmission. On the other hand the N_1 deflection was often unchanged concurrently with facilitation or inhibition of monosynaptic and polysynaptic reflexes. The conclusion is that the reticular points with inhibitory influence on the N_1 generating neurons do not coincide with those inhibiting motoneuron discharge.

Each reflex tested could be either inhibited or facilitated when different points were stimulated and from the same bulbar point some

reflex activity could be inhibited, some facilitated. The pattern of responses has changed practically from point to point and it does not seem possible to predict the type of response from the localization of the point stimulated. The great variance of the effect of stimulation has also been emphasized by GERNANDT and THULIN (1955).

On the assumption that the bulbar influence is exerted on post-primary neurons the conditioning effect upon the spinal cord dorsum response elicited from muscle afferents is consistent with earlier data (BERNHARD 1953) concerning the origin of the deflections. The small negativity following the afferent spike, considered as presynaptic, could not be influenced while the late negativity, presumably post-synaptic in origin, could be reduced to a great extent. The significance of the late cord dorsum negativity is so far little known. The fact that it has been found to undergo post-tetanic potentiation (LINDBLOM and OTTOSSON, unpublished observation) may imply that it represents monosynaptically transmitted activity (*cf.* LLOYD 1949).

Summary.

1. The influence of electrical stimulation of the bulbar reticular formation upon the cord dorsum response and ventral root reflexes elicited from cutaneous and muscle afferents has been studied in nembutalized cats.

2. Stimulation at certain points (see Fig. 2 E and F) resulted in a reduction of the negative cord dorsum potential evoked from low threshold cutaneous fibres (the N_1 deflection). The result is taken as an evidence of reticulospinal inhibition at the segmental sensory relay for transmission to suprasegmental levels of impulses from low threshold skin afferents.

3. Stimulation in other reticular points (see Fig. 4) resulted in a depression of the late cord dorsum response evoked from high threshold muscle afferents. The afferent spike and the early cord dorsum response evoked from low threshold muscle fibres were not influenced from any point.

4. The effects on the monosynaptic extensor reflex and the polysynaptic reflex elicited from a cutaneous nerve were studied for comparison. Inhibition of the N_1 deflection or the cord dorsum response evoked from high threshold muscle afferents and inhibition of the reflexes could be evoked from the same points or from different

points in the reticular formation. Further, one or both reflexes could be facilitated concurrently with depression of the cord potentials. It is not possible to predict the type of effect from the localization of the point stimulated.

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"In Vitro" Hemolysis of Erythrocytes from Vitamin E Deficient Rats and Chicks.

By

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and Ebbe Søndergaard.**

Received 28 July 1955.

GYÖRGY and ROSE (1949) have previously reported that dialuric acid, a reduction product of alloxan, induces rapid hemolysis, *in vivo* and *in vitro*, of the blood of vitamin E deficient rats. Tocopherol affords rapid and complete protection against this effect, apparently through its role as an antioxidant. These authors state "it is likely that it is some molecule or radical formed during the oxidation of dialuric acid that is the actual hemolyzing agent and which reacts with tocopherol". Other autoxidizable substances (ascorbic acid and sulfhydryl compounds such as cysteine and glutathione) at certain levels also were found to cause hemolysis, but the reaction was considerably slower than that with dialuric acid and required much larger amounts of reagent.

In later papers ROSE and GYÖRGY (1950, 1952) presented qualitative and quantitative procedures for studying the tocopherol requirement of rats based on this hemolysis. In the latter of these reports they state that hydrogen peroxide can be used in place of dialuric acid.

During the course of testing a large group of vitamin E deficient

[1] Visiting Fulbright Professor from Wesleyan University, Middletown, Connecticut, U.S.A.

rats by the dialuric acid hemolysis procedure, we observed that when suspensions of erythrocytes in isotonic saline solution were allowed to stand overnight at room temperature, considerable hemolysis resulted even in the absence of dialuric acid or other added autoxidizable substance. Accordingly it seemed of interest to study more fully the nature of this hemolysis. A few observations on blood from vitamin E depleted chicks are also reported.

Experimental.

Diets and animals.

The basal diet employed was a fat-free, tocopherol-free ration consisting of:

Casein, "low vitamin"[1]	20.0 %
Sucrose	74.3 %
Salt mixture[2]	5.0 %
Vitamin mixture[3]	0.5 %
Choline chloride	0.2 %
	100.0 %

One hundred and twenty eight female white rats were placed on this diet at about 3 weeks of age when they weighed approximately 25 grams. After 62 days most of the animals were shifted to other diets containing lard[4] at a level of either 2 % or 20 %. Some of these animals also were given d, *l*-α-tocopherol acetate (0.02 %) or methylene blue (0.126 %). All dietary substitutions were made at the expense of sucrose. All animals also received 4 drops per week of an aqueous "solution" of vitamins A and D, equivalent to 175 I.U. of vitamin A and 35 I.U. of vitamin D_3*.

The rats were kept in groups of four in roomy, raised-bottom wire cages. Food and water were supplied *ad libitum*.

A few experiments were performed on the blood of normal and vitamin E depleted chicks. All chicks were placed on the experimental diets containing about 30 % lard about 8 days after hatching and had been receiving

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[1] From Genatosan Ltd., Loughborough, Leics., England.

[2] McCollum-Simmonds salt mixture No. 185, supplemented with 0.131 % $CuSO_4$, 5 H_2O, 0.523 % $MnSO_4$, 4 H_2O and 0.013 % KI.

[3] Biotin 0.05 mg, folic acid 0.05 mg, ascorbic acid 5 mg, thiamine hydrochloride 5 mg, riboflavin 5 mg, pyridoxine hydrochloride 5 mg, calcium pantothenate 5 mg, nicotinic acid 8 mg, inositol 15 mg, p-aminobenzoic acid 35 mg, vitamin K (Synkavit, "Roche") 1 mg + sugar up to 500 mg.

[4] Some samples of lard were first dissolved in petroleum ether and passed through a column of "Filtrol" to remove the small amounts of vitamin E present, but this pre-treatment had no detectable effect on the hemolysis studies reported here.

* From A/S Ferrosan, Copenhagen.

these diets for 12 to 28 days. The depleted birds in all cases showed gross manifestations of severe vitamin E deficiency (encephalomalacia).

Dialuric acid hemolysis tests on rats.

Dialuric acid was prepared by the method of BAEYER (1863), recrystallized from boiling distilled water, and dried and maintained in a vacuum desiccator over concentrated sulfuric acid. The same preparation was used for all studies.

The hemolysis tests with dialuric acid were carried out by the method of ROSE and GYÖRGY (1950), slightly modified as follows:

> The equivalent of 2—3 large drops of blood from the snipped tail tip were collected in a 2 ml graduated centrifuge tube containing 1 ml of 0.9 % NaCl — 1.0 % Na citrate solution. After immediate mixing the tubes were centrifuged at 3 000 rpm for 10 minutes, the supernatant fluid was decanted and blotted off with filter paper, and the packed cells were resuspended in 1.5 ml of 0.9 % NaCl and thoroughly mixed. By micropipette 0.25 ml of the erythrocyte suspension was then added to each of two small hemolysis tubes, one containing 0.25 ml of phosphate buffer (pH 7.4), and the other containing 0.18 ml of buffer and 0.07 ml of 0.1 % dialuric acid in buffer. This gave a final concentration of red cells of approximately 1 % by volume. After mixing immediately, the tubes were placed in a water bath at 37° C for 15 minutes, then removed and held at room temperature.

The results obtained on 132 tests are listed in Table I. In no instance was hemolysis noted in any of the control tubes without added dialuric acid during the 4 hour observation period. Comparable tests were also performed at regular intervals on young male rats receiving a "normal" stock ration; of 26 animals so studied, 22 showed no dialuric acid hemolysis by the end of 4 hours, but 4 showed partial hemolysis.

Spontaneous hemolysis of rat erythrocytes in physiological saline.

It was observed early in our studies that when saline (0.9 %) suspensions of erythrocytes from vitamin E deficient rats were left standing overnight, moderate to strong hemolysis invariably was noted even in the absence of dialuric acid. Accordingly, a series of experiments was carried out to see how this hemolysis correlated with that induced by dialuric acid. To accelerate the process, higher temperatures were employed.

The blood samples were collected, centrifuged, and the cells re-

Table I.

Dialuric acid hemolysis observed with 132 female rats receiving various diets for different periods of time.

"Experimental" diet		Days on original fat-free diet without vit. E	Days on "experimental" diet	No. of rats tested	Number of rats showing hemolysis: Complete within 1 hour	Complete in 1–4 hours	Partial in 4 hours	None in 4 hours
fat-free	without vitamin E (same as original fat-free diet)	50	0	1			1	
		51	0	6		2	4	
		54[1]	0	12	12			
		51	0	13		13		
		58	0	12			7	5
		61[1]	0	12	3	6	3	
		62	0	12		7	5	
		84	0	2	1	1		
		97	0	2			2	
	+ methylene blue	62	23	2		2		
		62	35	2			2	
	+ vitamin E	62	25	2				2
		62	35	2				2
2% lard	without vitamin E	62	30	2	2			
		62	32	2	2			
		62	36	4		4		
	+ methylene blue	62	30	2		1	1	
		62	32	2			2	
		62	36	4			4	
	+ vitamin E	62	30	2				2
		62	32	2				2
		62	36	4				4
20% lard	without vitamin E	62	23	2	2			
		62	25	2				2
		62	29	4			3	1
		62	32	2	2			
		62	35	2			2	
	+ methylene blue	62	23	2	2			
		62	30	2			2	
		62	35	2			2	
	+ vitamin E	62	25	4				4
		62	29	4				4
		62	35	2				2

[1] The same rats were tested at a one-week interval.

suspended in 0.9 % NaCl as previously described, but the final dilution of the cell suspension was made with 0.25 ml of physiological saline instead of the dialuric acid and buffer solutions. The tubes were then kept immersed in a water bath at 37° C or 47° C and observed and tipped at regular intervals. The results are presented in Tables II and III. In these and later tables the following symbols have been used:

++++ = complete hemolysis
+++ = strong, incomplete hemolysis
++ = moderate hemolysis
+ = faint hemolysis
0 = no hemolysis

When purified nitrogen gas was bubbled through the red cell suspensions in saline for 30—46 seconds and thereafter the tubes were stoppered and incubated, no hemolysis occurred even after 24 hours.

Table II.

Spontaneous hemolysis of rat erythrocytes in physiological saline at 37° C. (All rats were pre-fed a fat-free, vitamin E-free diet for 62 days, then transferred to the diets listed below for 30—38 days before testing).

Diet	No. of animals	1 hour	Extent of hemolysis in	
			2 hours	4 hours
Fat-free.....	4	0	+	++ to +++
Fat-free + MB.....	4	0	0	+
Fat-free + vit. E...	4	0	0	0
2 % lard.....	8	0	+ to ++	++ to ++++
2 % lard + MB....	8	0	0 to +	+ to ++
2 % lard + vit. E...	8	0	0	0
20 % lard.....	6	0	+	+++ to ++++
20 % lard + MB...	4	0	0 to +	+
20 % lard + vit. E..	4	0	0	0

Hemolysis determination on blood of chicks.

To ascertain the applicability of hemolysis tests for studying vitamin E deficiency in the chick, blood specimens from 16 depleted birds and an equal number of normal controls were tested. The blood

Table III.

Comparison of the rate of saline hemolysis of rat erythrocytes at two different temperatures — 37° C and 47° C — carried out simultaneously.

Diet	1/2 hour		1 hour		2 hours		4 hours	
	37°	47°	37°	47°	37°	47°	37°	47°
Fat-free.....	0	++	0	++++	+		++	
Fat-free.....	0	++	0	++++	+		++	
Fat-free+MB...	0	+	0	+++	0		+	
Fat-free+MB...	0	+	0	++	0		+	
Fat-free+vit. E .	0	0	0	0	0		0	
Fat-free+vit. E .	0	0	0	0	0		0	
2 % lard.....	0	+	0	++++	++	++++	+++	
2 % lard.....	0	+	0	++++	+	++++	+++	
2 % lard.....	0	0	0	+++	+	++++	+++	
2 % lard.....	0	0	0	+++	+	++++	++	
2 % lard+MB ..	0	0	0	++	+	+++	+	
2 % lard+MB ..	0	0	0	++	0	+++	+	
2 % lard+MB ..	0	0	0	+	0	+++	+	
2 % lard+MB ..	0	0	0	+	0	+++	+	
2 % lard+vit. E.	0	0	0	0	0	0	0	
2 % lard+vit. E.	0	0	0	0	0	0	0	
2 % lard+vit. E.	0	0	0	0	0	0	0	
2 % lard+vit. E.	0	0	0	0	0	0	0	
20 % lard.....	0	++	0	++++	+		+++	
20 % lard.....	0	++	0	++++	+		+++	
20 % lard+vit. E	0	0	0	0	0		0	
20 % lard+vit. E	0	0	0	0	0		0	

(0.2 ml) was withdrawn by syringe from the jugular vein into saline-citrate solution, centrifuged, and the cells resuspended in 0.9 % NaCl solution in the manner previously described for rats.

Table IV shows the extent of hemolysis induced by dialuric acid when (A) the red cell suspensions were incubated for only 15 minutes at 37° C and then held at room temperature thereafter, and (B) when the cell suspensions were maintained in a 37° C bath for 4 or 6 hours before being removed to room temperature. An equal number of control chicks which had received vitamin E supplemented diet were also tested concurrently. These are not listed in the table, but in no instance was hemolysis observed under the conditions corresponding to (A) above. Under conditions corresponding to (B) faint hemolysis sometimes occurred by the end of 24 hours.

Table IV.

In vitro hemolysis by dialuric acid of erythrocytes from chicks deficient in vitamin E.

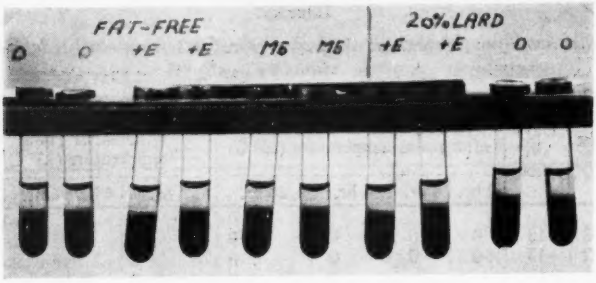
Chick no.	Days on diet	A. 37° C for 15 min., thereafter room temperature (23° C)				B. 37° C for 4–6 hrs., thereafter room temperature (23° C)			
		2 hr.	4 hr.	6 hr.	24 hr.	2 hr.	4 hr.	6 hr.	24 hr.
2668	13	0	0	0	+				
2672	13	0	0	0	++				
2811	12					0	0	+	+
2813	12					0	0	0	+
2785	12					+	+	++	++
2807	12					0	+	+	+
2785	14	0	0		+	0	+		+
2783	14	+	+		++	++	++		++
2786	14	0	0	0	0	0	0		+
2779	27	0	0	0	+	0	0	0	+
2784	27	0	++	+++	+++++	++	++	++	++
2777	27	0	+	+	++	+	+	+	+
2782	27	0	0	0	+	0	0	0	0
2797	28	0	++		+++				
2808	28	0	++		++				
2815	28	0	0		+				

Discussion.

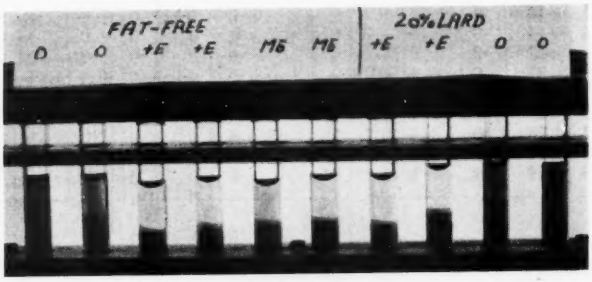
The results obtained with the blood of vitamin E deficient rats demonstrate clearly that *in vitro* hemolysis can occur in the absence of any added autoxidizable substance, although the presence of the latter will accelerate the process at low temperatures. Suspensions of washed red cells of E-deficient rats in physiological saline alone will hemolyze extensively when allowed to stand at room temperature overnight. When they are incubated at somewhat higher temperatures the rate of spontaneous breakdown is increased considerably. Thus, at 37° C moderate to complete hemolysis is observed within 4 hours, while at 47° C hemolysis is usually complete by the end of one hour (see Figure 1 and Table III).

That temperature is an important factor in the rate with which human red cells are hemolyzed by hydrogen peroxide has recently been noted by HORWITT, DUNCAN, and HARVEY (1955).

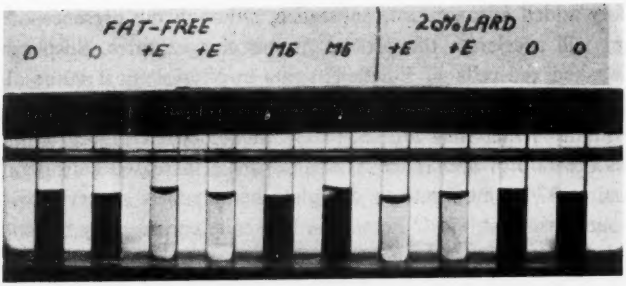
Erythrocytes from vitamin E deficient rats were no more sensitive to hemolysis by hypotonic solutions than were those of normal controls when examined by the usual osmotic resistance test.



A. Room temperature (23° C) for 5 1/2 hrs.



B. 37° C for 3 1/2 hrs.



C. 47° C for 1 hr.

Fig. 1. Showing the effect of temperature on the rate of hemolysis in isotonic saline of the erythrocytes from vitamin E deficient rats. The complete protection afforded by vitamin E (+ E) and the partial protection given by methylene blue (Mb) are especially evident in "B".

The results that we obtained using dialuric acid (Table I) were not as consistent as we had anticipated. Animals on the vitamin E-free diets containing either no fat or 20% lard showed great differences in their hemolytic response, from complete hemolysis in less than 1 hour to no detectable hemolysis within a 4 hour period. Once the same group of 12 rats was tested twice, after 54 days and 61 days on the fat-free and E-free diet, with quite different results. In every instance, however, the inclusion of α-tocopherol in the diet protected completely against hemolysis, and the addition of methylene blue to the E-free diets afforded clear-cut partial protection in most cases. This confirms the previous report in this connection by CHRISTENSEN and DAM (1951).

The data obtained on the spontaneous hemolysis in isotonic saline at 37° C (Table II) were much more consistent. Hemolysis began after incubation for 1—2 hours, and by the end of 4 hours it ranged from moderate to complete. Dietary methylene blue again gave marked but incomplete protection, whereas dietary tocopherol protected completely.

In view of the above results the question arises as to why hemolysis does not occur *in vivo* in vitamin E-deficient rats.

The erythrocytes of chicks on an encephalomalacia producing diet, in contrast to those of rats on various vitamin E-free diets, are very resistant to hemolysis. When suspended in isotonic saline and kept at 37° or 47° C for 7 hours and then at room temperature overnight, no hemolysis of the chick cells occurred. When dialuric acid was included, the cells of 8 out of 16 grossly deficient chicks showed no trace of hemolysis after 4 hours, and the others showed only faint to moderate hemolysis (Table IV). Even after 24 hours only two chick bloods showed strong hemolysis. The higher incubation temperature caused hemolysis to begin a little sooner, but the extent achieved by the end of 24 hours was about the same at room temperature (23° C) as at 37° C. There was no apparent correlation between the degree of hemolysis of the chick erythrocytes and the length of time they had been on the experimental diet, nor did hemolysis correlate with the presence or degree of clinically manifest encephalomalacia. Consequently, we feel that the dialuric acid method is not satisfactory for assessing tocopherol deficiency in the chick. Perhaps the fact that the erythrocytes of birds are nucleated, living cells contribute to their greater resistance to oxidative hemolysis.

Summary.

When red blood cells of rats deficient in vitamin E are suspended in isotonic (0.9%) NaCl solution and allowed to stand overnight at room temperature, extensive spontaenous hemolysis occurs. This oxidative process is accelerated by incubating the suspensions at higher temperatures, hemolysis being nearly complete within one hour at 47° C.

Hemolysis did not occur when purified N_2 had been bubbled through the erythrocyte suspension in saline to remove free O_2.

Although dialuric acid increases the rate of hemolysis, the presence of this substance, hydrogen peroxide, or other added autoxidizable compound or oxidizing agent is not essential to the process.

The incorporation of small amounts of methylene blue in the diet of vitamin E deficient rats affords marked but incomplete protection against *in vitro* hemolysis.

Erythrocytes from tocopherol-depleted chicks (on an encephalomalacia producing diet) manifesting severe deficiency symptoms are much more resistant to hemolysis than are those from rats. Spontaneous hemolysis of chick red cells in saline solution was not noted within 24 hours even when the cell suspensions were incubated at temperatures up to 47° C for 7 hours. The addition of dialuric acid caused faint to moderate hemolysis within 4 hours in about half of the birds studied.

It is concluded that whereas the *in vitro* hemolysis of red cells may be used for assessing vitamin E deficiency in the rat, it is not a reliable criterion for determining a deficiency of this vitamin in the chick.

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On the Emotional Eosinopenic Reaction Caused by Examination.

By

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It is known that lately, since CANNON (1915), more attention has been paid to the importance of the emotions in our somatic functions in clinical medicine as well in our daily life. The exact definition of the "emotion" is not easy. According to COBB (1950) the "emotion" is an introspectively given subjective state, usually mediated by acts of interpretation, the whole set of internal physiological changes, which help to return the normal equilibrium between the organism and its environment, and the various patterns of behavior, stimulated by the environment and implying constant interactions with it and also the more or less agitated psychological state. An emotion is a disturbance, involving somatic changes, which is experienced as a more or less agitated feeling. Both the feeling and the behavior which expresses it, as well as the internal physiological responses to the stimulus situation, constitute a dynamically interrelated whole. To the positive or negative emotion further belongs a series of chemical and physiological changes in the individual, the neurological mechanisms in the visceral brain and in the autonomic nervous system, and

a more or less typical behavior as the manifestation of a certain affect. Further it appears in connection with a specific psychological state, as for instance anger, fear, sorrow, excitement, joy and love.

Attempts have been made to correlate the physical and psychic strain, the fear and excitement caused by the examination, and the function of the autonomic nervous system. In connection with the examination there may appear, especially with vegetatively labile individuals, the same emotional symptoms, as when a person gets an injection of adrenalin, as for instance feelings of fear and excitement, palpitation of the heart, trembling, a slight rise in the blood pressure, paleness, and glucosuria. CANNON (1915, 1932) has paid attention to the emotional reactions and the autonomic nervous system in the animal during experiments through the influence of fear or anger. He has shown the importance of the autonomic nervous system in the emergency reactions, *e.g.* in a state of fear, when there can take place a secretion of adrenalin or sympatin. Nowadays, according to V. EULER (1950), sympatin most closely corresponds to noradrenalin. CANNON's (1915) observations in the autonomic nervous system form the experimental basis of our knowledge on the humoral mechanism of the emotional reactions in the organism.

It has also been shown the importance of the hormonal secretion from the adrenal cortex for the adaptation phenomena of the organism. LONG and FRY (1945), THORN, FORSHAM, PRUNTY and HILLS (1948), THORN (1952), and LONG (1947, 1952) have pointed out that adrenalin through the anterior pituitary gland can activate the function of the adrenal cortex, which in turn causes a decrease in the number of the eosinophil cells in the blood.

The emotional eosinopenic reaction caused by examination may be one of the physiological manifestations of the acute negative feeling of fright and tension, but our knowledge of the nature of this type of eosinopenia is relatively scanty.

In earlier investigations RENOLD, QUIGLEY, KENNARD and THORN (1951) and WAKE, GRAHAM and MAC GRATH (1953) noticed the eosinopenic reaction in connection with competition anxiety or heavy physiological exercise. As to the excitement during an examination HUMPREYS and RAAB (1950) and DREYFUSS and FELDMAN (1952) have ascertained that the number of eosinophil cells in the blood decrease to almost one half. There has after that been scanty knowledge of the examination eosinopenia.

The Purpose of the Investigation.

We have studied the appearance of the emotional eosinopenic reaction in the blood in connection with examinations during the years 1951—54 in female and male students, taking into account the strength, duration and probability of the eosinopenic reaction, and individual fluctuations of the number of the eosinophil cells in the blood. Although the physical and psychic exertion caused by the preparing for the examination might be lasting a long time before, the real moment of tension coincides with the period, when the examination questions are being read. In accordance with THORN's adrenalin and ACTH tests (1952), the eosinophil counts were taken in a written examination about 4—5 hours after the reading of the questions and in an oral examination approximately 2 hours after the beginning of it.

Material and Methods.

The material has been gathered from altogether 10 groups of examinations, of which 6 were final examinations in Medical Biochemistry (the groups I—V and X) and 4 such ones in the Faculties of Natural Science and Arts (the group VI—IX). The examinations were held with altogether 93 students of Medical Biochemistry and 19 studying Natural Science and Arts. 33 of them have been female and 79 male students.

Cell counts were taken from 78 students in Medical Biochemistry (the groups I a—V a and I b—V b) after the examination and in 44 students in addition during the non-stress period, usually 1—2 weeks after the examination. Tests were taken from other 34 students of Medical Biochemistry (the groups I b—V b) only during the examination, but not owing to the volontary nature of the test, later on during the examination-free time.

From 15 students of Medical Biochemistry and 19 students at the faculty of Science (the groups VII and IX) and Arts (the groups VI and VIII) tests were taken also two to three successive days before and immediately before, and immediately after the examination and two to three days after it.

The eosinophil cells have been counted from capillary blood, tests taken from the tip of the finger, in dilution to 1:11 with a solution containing 100 mg. phloxine B in 100 ml. propyleneglycol and water mixture aa according to RANDOLPH (1949). After the careful mixing the solution was pipetted in a Fuchs-Rosenthal counting chamber. The cells were allowed to sink to the bottom of the counting chamber during 15—30 minutes before counting cells. The cells were counted in 2—4 specimens.

Results.

Eosinophil Counts in Individual Cases and in Examination Groups (Table I).

The individual decreases of the eosinophil cell numbers in the blood during examination in 78 students of Medical Biochemistry (the groups 1 a—V a and I b—V b) are described in Table 1-compared to the eosinophil number during the non-stress period. In almost every student the examination decreased the number of eosinophil cells in the blood, but in varying amounts, in 9 students to 0, and in few only little. In those students, in which the cell number has been more than 150 per cm, the decrease of the absolute cell number has been larger than in students, in which the cell number has been under 100 per cmm. In 34 students (the groups I b—V b) for which there were no cell counts from an examination-free period, the eosinophil counts are marked only during the examination.

As compared to the eosinophil counts of the control period the average decrease during the examination for the groups I—V can be seen in table 1. During the examination-free period the number of eosinophil cells in the student groups II, IV and V are more or less normal (156, 198 and 167 cells per cmm blood respectively), but in the two groups I and III rather low-115 and 121 cells per cmm respectively.

The eosinophil numbers have decreased during the examination in all these groups to more than $^1/_2$ of the control count. In groups I a—V a immediately after the examination in 44 students the eosinophil number has been 28, 69, 64, 89, and 25 cells per cmm blood respectively. In average during the non-stress period there were 155 eosinophil cells per cmm blood and immediately after the examination 56.

From those 34 students who did not come to the control test during the non-stress period, the average cell counts during the examination are shown in the table I. In these groups (I b—V b) the average eosinophil number immediately after the the examination has been 70, 63, 59, 48 and 46 cells per cmm blood, respectively.

In the whole material—in addition it also comprised the 34 cases in which the number of eosinophil cells in the blood were followed during several days before and after the examination (the groups VI—X)—the eosinophil cell count during the control time was 157

Table I.

The Decrease of the Number of Eosinophils in Blood during the Examination as compared to the Examination-free Period in Groups of 78 Medical Students. Individual Cases. Groups I—V.

Group	No.	Sex	Eosinophils pr cmm of blood: Non-stress Period	Eosinophils pr cmm of blood: After Examination	Decrease in %
I a	1	♀	180	0	−100
	2	♀	60	0	−100
	3	♂	48	36	− 25
	4	♂	131	84	− 36
	5	♂	156	18	− 88
	Average		115	28	− 70
II a	6	♂	241	120	− 50
	7	♀	175	72	− 59
	8	♀	471	203	− 57
	9	♂	86	41	− 52
	10	♀	76	24	− 68
	11	♂	38	17	− 55
	12	♂	289	69	− 76
	13	♀	127	48	− 62
	14	♂	31	34	+ 10
	15	♀	31	10	− 68
	16	♂	175	65	− 63
	17	♀	138	124	− 10
	Average		156	69	− 51
III a	18	♂	124	28	− 77
	19	♂	134	96	− 28
	20	♀	45	38	− 15
	21	♂	131	45	− 66
	22	♂	103	65	− 37
	23	♂	186	110	− 41
	Average		121	64	− 44
IV a	24	♂	523	174	− 67
	25	♂	72	34	− 53
	26	♂	113	104	− 8
	27	♀	118	38	− 68
	28	♂	289	65	− 78
	29	♀	65	14	− 78
	30	♂	327	217	− 34
	31	♀	79	62	− 22
	Average		198	89	− 61
V a	32	♂	146	0	−100
	33	♂	146	0	−100
	34	♂	73	12	− 84
	35	♂	122	104	− 15
	36	♂	293	37	− 87
	37	♂	232	24	− 81
	38	♂	73	0	−100
	39	♂	73	0	−100
	40	♂	206	55	− 73
	41	♂	317	30	− 90
	42	♂	98	0	−100
	43	♂	176	44	− 75
	44	♀	165	22	− 87
	Average		167	25	− 82

Group	No.	Sex	Eosinophils pr cmm of blood: Non-stress Period	Eosinophils pr cmm of blood: After Examination	Decrease in %
I b	45	♀	—	168	+ 8
	46	♀	—	36	− 77
	47	♂	—	6	− 96
	Average			70	− 55
II b	48	♂	—	86	− 44
	49	♂	—	27	− 82
	50	♂	—	155	0
	51	♂	—	31	− 80
	52	♂	—	86	− 44
	53	♂	—	96	− 32
	54	♂	—	17	− 89
	55	♂	—	89	− 43
	Average			63	− 52
III b	56	♂	—	96	− 38
	57	♂	—	55	− 65
	58	♂	—	14	− 91
	59	♂	—	62	− 60
	60	♂	—	100	− 35
	61	♂	—	28	− 82
	Average			59	− 62
IV b	62	♂	—	28	− 82
	63	♂	—	24	− 84
	64	♀	—	48	− 70
	65	♀	—	66	− 58
	66	♀	—	62	− 60
	67	♀	—	52	− 67
	68	♀	—	48	− 70
	Average			48	− 70
V b	69	♂	—	143	− 8
	70	♂	—	77	− 50
	71	♂	—	5	− 93
	72	♂	—	0	−100
	73	♂	—	0	−100
	74	♂	—	48	− 70
	75	♂	—	66	− 58
	76	♂	—	24	− 84
	77	♂	—	60	− 61
	78	♂	—	44	− 72
	Average			46	− 70

cells per cmm in 78 students and immediately at the end of the examination 64 cells per cmm in 112 students.

Duration and Strength of Eosinopenic Reaction.

In order to ascertain the duration and strength of the eosinopenic reaction in connection with the examination (the groups VI—X) the eosinophil cells in the blood were counted also during 2—3 days before and after the examination in 34 students (Table 2).

The eosinophil count became gradually lower during three days until the examination was over. It fell during three days before the examination from 153 to 128 (group VI), from 148 to 97 (group VII), from 219 to 174 (group IX), and from 130 to 101 (group X) per cmm blood and continued to fall, rapidly first during the examination. Immediately after the examination the eosinophil number is clearly smaller than before the examination and the reading of the questions. *This shows that the eosinopenia depends on the examination moment.* In group VI the cell number dropped during the examination to 48, in group VII to 61, in group IX to 107, and in group X to 76 cells per cmm blood. In group VIII there is no decrease in the number of the eosinophil cells after the examination; in that group the cell count was already 3 days before the examination low, but one day before it increased. If we compare with that latter figure, a decrease can be seen even here. After the examination the eosinophil count rises relatively swiftly in 1—3 days to its original level.

The decrease in the number of eosinophil cells lasts a short time. When we take into account that the examination often required a months preparation, the eosinopenic reaction due to the examination must be considered as a phenomenon of a relatively short duration. It lasts only during the examination and begins 1—2 days before it; but even one day before examination the decrease of the number of eosinophil cells may be slight.

Percentual Changes of the Eosinophil Counts.

When the decrease in the number of eosinophil cells is expressed in per cent (Table 1), it can be ascertained that in all the groups of students (I—X, with the exception of group VIII), the eosinophil cells have decreased during examination with about 50 % (70, 51, 44, 61, 82, 68, 62, 11, 57, and 49 % respectively).

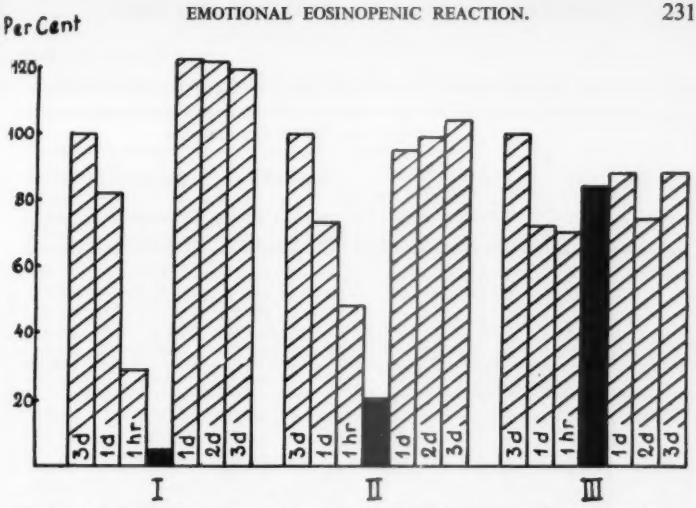

Fig. 1. Individual Eosinopenic Reactions of Three Students as Percentage during three Days before and after the Examination. Examination is marked with the dark column.

Average decrease of eosinophil cells has been in these groups 63 per cent. In those cases for which there were no cell counts from an examination-free period, the decrease in the eosinophil number has been shown in per cent, by choosing the average eosinophil count of the other medical students from an examination free period as basis.

This decrease in eosinophil cells is equally big as after the subcutaneous injection of the 0.3—0.5 mg. adrenalin or after the intramuscular injection of 25 mg. of ACTH.

When according to THORN's test the decrease in the number of eosinophil cells in individual cases is expressed as percentage (Table 1), 9 of these students have had a 100 % decrease in the number of eosinophil cells in the blood, 54 students more than 50 % and 24 students under 50 % decrease, and only in a few it was relatively small. The percentage had in two case even slightly increased.

In order to make clear the eosinopenic reactions ascertained in different individuals we have presented 3 students, in which the decrease of the eosinophil cells in the blood shows three typical reactions (figure 1). In the first and second student the decrease of the eosinophil cells is clear and takes place gradually, but in the third student there is no decrease in the cell count.

Table

The Absolute Number of Eosinophils during three Days before, immediately

Group	No.	Sex	Before Examination				
			3 Days before	1 Day before		Immediately before	
			Number per cmm	Number per cmm	Change in %	Number per cmm	Change in %
VI	79	♂	218	178	− 18	130	− 40
	80	♂	161	169	+ 5	116	− 18
	81	♂	114	81	− 29	91	− 20
	82	♂	51	87	+ 70	67	+ 32
	83	♀	222	122	− 45	112	− 50
	Average		153	128	− 3	103	− 19
VII	84	♀	178	128	− 18	124	− 30
	85	♀	81	59	− 17	39	− 52
	86	♂	191	106	− 45	189	− 1
	87	♀	142	92	− 35	63	− 56
	Average		148	97	− 29	104	− 35
VIII	88	♀	188	209	+ 10	130	− 31
	89	♂	70	81	+ 15	67	− 4
	90	♂	103	182	+ 77	102	− 1
	91	♂	80	114	+ 43	56	− 30
	92	♂	121	192	+ 59	293	+143
	Average		112	156	+ 41	131	+ 15
IX	93	♂	308	122	− 60	400	+ 30
	94	♂	195	258	+ 33	214	+ 10
	95	♀	17	44	+158	8	− 53
	96	♀	344	256	− 26	216	− 37
	97	♀	232	189	− 18	69	− 70
	Average		219	174	+ 17	182	− 24
X	98	♂	89	55	− 38	58	− 35
	99	♂	101	125	+ 24	59	− 42
	100	♂	202	136	− 32	−	−
	101	♀	275	275	0	234	− 15
	102	♀	45	20	− 55	−	−
	103	♂	132	112	− 15	69	− 48
	104	♂	61	69	+ 13	69	+ 13
	105	♀	83	92	+ 11	42	− 49
	106	♂	74	36	− 51	−	−
	107	♂	109	72	− 36	101	− 7
	108	♂	250	144	− 42	−	−
	109	♀	175	163	− 7	−	−
	110	♂	88	30	− 66	−	−
	111	♂	52	106	+104	98	+ 89
	112	♀	214	95	− 56	−	−
	Average		130	101	− 22	91	− 12

II.

before and after, and two to three Days after Examination.

After Examination							
Immediately after		1 Day after		2 Days after		3 Days after	
Number per cmm	Change in %	Number per cmm	Change in %	Number per cmm	Change in %	Number per cmm	Change in %
50	− 77	—		—		—	
83	− 48	—		—		—	
28	− 75	—		—		—	
14	− 72	—		—		—	
67	− 70	—		—		—	
48	− 68	—		—		—	
141	− 21	156	− 12	128	− 28	156	− 12
16	− 80	77	− 5	80	− 2	88	+ 4
31	− 84	102	− 47	263	+ 38	114	− 40
56	− 61	100	− 29	128	− 10	95	− 33
61	− 62	109	− 23	150	− 1	113	− 20
142	− 24	202	+ 7	238	+ 27	206	+ 9
31	− 56	80	+ 14	82	+ 17	58	− 17
156	+ 52	75	− 29	100	− 3	45	− 56
28	− 65	59	− 26	66	− 17	63	− 21
238	+ 97	242	+100	242	+100	447	+270
117	+ 1	131	+ 14	146	+ 25	163	+ 37
277	− 10	263	− 15	278	− 10	286	− 8
97	− 50	131	− 33	197	+ 1	—	—
5	− 71	6	− 65	14	− 17	—	—
144	− 58	182	− 47	164	− 52	119	− 65
14	− 94	283	+ 22	283	+ 22	275	− 19
107	− 57	173	− 27	187	− 11	227	− 11
—	—	70	− 21	55	− 38	—	—
—	—	121	+ 20	138	+ 37	—	—
81	− 60	164	− 19	122	− 39	—	—
—	—	252	− 9	244	− 11	—	—
14	− 69	70	+ 56	25	− 44	—	—
—	—	83	− 37	96	− 18	—	—
—	—	—	—	166	+172	—	—
—	—	76	− 8	31	− 63	—	—
25	− 66	44	− 41	66	− 11	—	—
—	—	102	− 6	46	− 58	—	—
217	− 13	—	—	248	− 1	—	—
86	− 51	207	+ 19	195	+ 12	—	—
94	+ 7	47	− 47	54	− 39	—	—
—	—	253	+388	403	+677	—	—
16	− 92	178	− 17	166	− 12	—	—
76	− 49	128	+ 12	136	+ 38	—	—

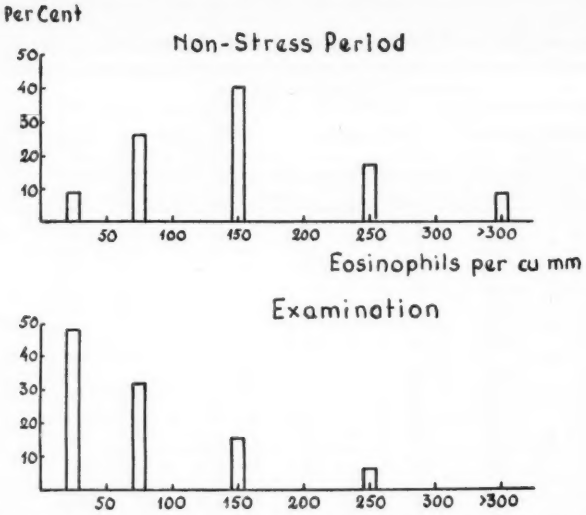

Fig. 2. Distribution of the Number of Eosinophils during the Non-stress Period and immediately after the Examination in the Whole Material (as Percentage).

Individual Variation of Counts during the Examination-free Period and during the Examination.

100—350, in average 185 eosinophil cells per cmm of blood are often considered normal. In figure 2 we see the percentage grouping of the eosinophil cell counts during the control period and the examination time in the whole material of 112 students. During the control period the eosinophil count was under 100 cells per cmm in 34 per cent of the students and less than 50 cells per cmm in 8 per cent. Immediately after the examination there were less than 100 cells per cmm in 80 per cent and less than 50 cells per cmm in 48 per cent of the students.

Following the changes in the eosinophil cell counts in female and male students we can in the whole material observe that the counts decreased in average a little more with women than with men. The difference is, however, not of any statistical significant. In some groups the women have a distinctly stronger eosinopenic reaction than the men. In the group of medical students, in which less speci-

mens were taken from women than from men students, no difference was ascertained in the reaction.

Discussion.

It is possible to assume that the eosinopenic reaction in connection with an examination can be due either to an endogenous secretion of adrenalin from the adrenal medulla or to the secretion of adrenocortical hormones caused by the adrenocorticotrophic hormone of the pituitary gland through the impulses from the hypothalamus or both. The reflex secretion of adrenalin caused by fear and excitement in test-animals, the changes in the carbohydrate metabolism, the subjective neurovegetative and cardiovascular symptoms observed during the examination indicate that adrenalin possibly plays an important part in it, as they are changes typical for adrenaline just as the decrease in the eosinophil cells in the blood. Noradrenalin which is a mediator in the sympathetic nervous system and a part of adrenal medulla, can also reduce the number of the eosinophil cells in the blood, but much less than adrenalin (HUMPREYS and RAAB, 1950). — The mechanism of the eosinopenic reaction during the examination, however, is not clear. It can partly be solved by determining whether the amount of the adrenalin, noradrenalin or adrenocortical hormones in urine or in the blood simultaneously is increased.

In connection with examination the specimens have generally been taken 4—5 hours after the reading of the questions, at which moment, according to THORN's test, we can expect the biggest decrease in the eosinophil cells. As the percentage changes more or less correspond to those of THORN's test, it is likely that the blood specimens in connection with the examinations have been taken at the moment of the maximum reaction.

In some cases the decrease in the number of eosinophil cells is strong, in other cases their number is stable or almost the same in spite of the examination. The number of the eosinophil cells varies already by itself considerably in different individuals and partly also according to the day and night rhytm (BONNER 1952, FLINK and HALLBERG 1952). The eosinophil count decrease before noon 20 per cent. Additional investigations will show whether the number of eosinophil cells is labile from one day to another, and whether it, on the other hand, remains unchanged and almost stable from one day to another even during a long time of observation.

Investigations give reason to assume that there is a certain correlation between the psychic factors of the examination and the eosinopenic reaction of the blood, which correlation can be elucidated in part by careful psychoanalysis. But many other factors influence upon the number of eosinophil cells in daily life. This is indicated among other things by these tests, in which at least in one case the reaction has not appeared, although the person in question has subjectively been afraid of the examination. In this case the number of eosinophil cells has, however, been relatively high, on the third day after the examination it was even 447 cells per cmm. The individual decrease can also depend on the severity of the examination stress and on psychological factors. At an examination the physical performance is relatively small and the psychic strain dominates.

The adrenalin diminishes the eosinophil count also in the blood of a rat and mouse, but not after the adrenals or the pituitary gland has been taken away (LONG and FRY 1945, LONG 1947, GERSCHBERG, FRY, BROBECK and LONG 1950, MC DERMOTT, FRY, BROBECK and LONG 1950, STEIN, MARTIN and FALK 1951, HUME and WITTENSTEIN 1950, SCHWEIZER 1953). A certain correlation has been ascertained between the eosinophils in the blood and the cholesterol content in the adrenals (MC DERMOTT, FRY, BROBECK and LONG 1950), but, on the other hand the ascorbic acid in the adrenals and the eosinophil cells in the blood need not always be parallel phenomena. The adrenalin eosinopenia may be due to the secretion of cortisone or adrenal cortex hormone (HENRY, OLIVER and RAMEY, 1953). It has also been ascertained that adrenalin can stimulate the adrenal cortex, when the pituitary gland has been taken away (VOGT 1944). Adrenalin may cause eosinopenia in human beings even after the adrenals have been taken away (THORN 1952, HENRY, OLIVER and RAMEY 1953, MC JEFFRIES, LEVY and KELLY 1953). Big doses (1.5 mg.) of adrenalin can cause an eosinopenic reaction in patients who suffer from the ADDISON's disease while small doses (0.3 mg.) almost never, or at least very seldom cause a positive reaction (WALDENSTRÖM 1950). The adrenocorticotrophic hormone does not cause any eosinopenia after the adrenalectomy, but on the contrary it increases the eosinophil number (DUMM and RALLI 1954).

It has been observed the secretion of the adrenocorticotrophic hormone also by direct application of adrenalin to pituitary grafts (MCDERMOTT, FRY, BROBECK and LONG 1950). The hypothalamus-

pituitary gland system is according to the recent findings relatively insensitive to activation by adrenalin (MEYER 1953). Although adrenalin causes eosinopenia, it does not increase the level of 11-oxisteroids in the blood, nor in the adrenal vein (BLISS, NELSON and SAMUELS 1954, NELSON, SANDBERG, PALMER and GLENN 1952, LABHART and SPENGLER 1953) or the level of the adrenocorticotrophic hormone in the blood (GEMZELL 1955). Thus adrenalin seems to have a direct influence upon the eosinophil cells without the pituitary adrenal system.

The hypothalamus may possibly secrete adrenergic substance into the hypothalamo-hypophysial portal system and in such a way stimulate the anterior pituitary gland to secrete adrenocorticotrophic hormone in the blood (LAST, JORDAN, PITESKY and SIEGEL 1950). Nembutal (RECANT, HUME, FORSHAM and THORN 1950) and other barbiturates (SAYERS and SAYERS 1948), which are central nervous system depressants, prevent the eosinopenic reaction of the adrenals probably selectively by influencing upon the hypothalamus. Adrenalin and noradrenalin have been found in the region of the hypothalamus (VOGT 1953).

Summary.

The appearance of the eosinopenic reaction in the blood in connection with examinations in 33 female and 79 male students have been studied, taking into account the strength, probability and individual fluctuations of the reaction.

In average there were during the non-stress period 157 cells per cmm blood in 78 students and immediately after the 4—5 hours examination an average of 64 cells in 112 students. The average cell counts of the eosinophil cells have decreased during the examination in all these groups, in average to more than half of the normal. In all the examination groups with one exception the eosinophil cells have decreased with about 50 % (70, 51, 44, 61, 82, 68, 62, 57, 11 and 49 % respectively). During the control period the eosinophil count was under 50 cells per cmm only in 8 %, but immediately after the examination there were less than 50 cells per cmm in 48 % of cases. In almost every students the examination decreased the number of eosinophil cells, in some to a very great extent, but in few only little. 9 students have had a 100 % decrease in the eosinophil cells in the blood after examination.

In order to ascertain the duration and strength of the eosinopenic reaction in connection with an examination the eosinophil cells in the blood were counted also during three days before and after the examination in 34 students. The eosinophil cell count became gradually lower during three days, and continued to fall till the examination began. One hour before the examination the average decrease of number of eosinophil cells was in these students 15 per cent and immediately after the examination 47 per cent. In these groups the decrease in the number of the eosinophil cells in the blood was strongest on the examination day immediately after the examination. *This shows that the eosinopenia depends on the examination moment.* After the examination the cell count rises relatively swiftly in 2—3 days to its control level.

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Measurement of the Extracellular Fluid Volume by Thiosulfate.

I. The measurement of the apparent volume of distribution of thiosulfate.

By

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Many methods have been presented for the measurement of the extracellular fluid volume in man (TURNER and LEVITT 1953, MCCANCE 1955). CARDOZO and EDELMAN (1952) introduced a method employing thiosulfate, which in comparison with most of the others offers the advantages of being simple and rapid.

In a previous work from this laboratory it was shown that the volume of distribution of thiosulfate was approximately the same as the volume of distribution of inulin (IKKOS 1955).

The aim of the present work was to examine further the validity of the thiosulfate method. In the present part the accuracy and reproducibility of the measurements of the thiosulfate space are examined. In another part (IKKOS *et al.* 1956) the apparent volume of distribution of thiosulfate is compared with the inulin space.

Materials and Methods.

The material comprises 24 subjects, 15 males and 9 females, ranging in age between 20 and 54 years. Thirteen (cases 1—12 and 20) were normal adults or patients treated for minor disorders without obvious or probable changes in the metabolism or distribution of water (group I). Seven of the remaining subjects (cases 13—19) were patients treated for metabolic

diseases (group II). Of the latter group, 4 had acromegaly, 2 had Cushing's syndrome and one was a hypophysectomized cancer patient. Finally, group III (cases 21—24) comprises three hypophysectomized diabetic patients and one man suffering from Cushing's syndrome. These patients were all known to have renal damage.

The measurement of the thiosulfate space was performed according to the method of CARDOZO and EDELMAN (1952). Fifty to 100 ml of a 10 per cent solution of anhydrous sodium thiosulfate were given intravenously at an approximately constant rate over a period of 8 to 10 minutes. The injections were given from calibrated pipettes, and the connection to the needle carefully washed with saline at the end of the infusion. Venous blood samples were drawn usually 20, 30, 40, 50, 60 and 70 minutes after zero time, from an indwelling needle with the smallest possible stasis applied to the arm. The time of the sampling was noted to the nearest half-minute.

The concentration of the thiosulfate in the solution given was not measured for every test, but samples of every batch of thiosulfate solution were analyzed from time to time. The concentration of the thiosulfate injected varied between 9.94 and 10.12 g per cent with a mean value of 10.03 g per cent. In the calculations of the space the concentration of the thiosulfate injected was taken as exactly 10 grams per 100 ml.

In cases 1—19 double measurements of the thiosulfate space were performed on two different occasions. In case 20 four measurements were performed within one month, and a fifth measurement one year later. In cases 21—24 a catheter was inserted into the bladder and urine specimens for thiosulfate analysis were obtained during the test. The bladder was rinsed with saline and emptied with air at the end of each period of urine collection.

The analytical method used for thiosulfate in plasma was the indirect iodometric macromethod of NEWMAN *et al.* (1946), and for thiosulfate in urine, the direct macromethod of the same authors.

The accuracy of the measurement of thiosulfate in plasma was calculated from double determinations on the same plasma in 38 samples. The mean difference was found to be 0.20 mg per cent. This value agrees well with that found when duplicate titrations were run on the same plasma filtrate (0.15 mg per cent). The recovery of thiosulfate from plasma varied between 98 and 104 per cent with a mean value of 101.2 per cent.

The thiosulfate space was calculated by the formula

$$V_{TS} = \frac{TS_{\text{inj}}}{C_0} \quad \ldots\ldots\ldots\ldots (1)$$

where V_{TS} is the apparent volume of distribution of thiosulfate in liters, TS_{inj} the amount of thiosulfate injected in mg, and C_0 the concentration of thiosulfate in plasma in mg per liter at zero time (t_0). After transformation into logarithms we obtain

$$\log V_{TS} = \log TS_{\text{inj}} - \log C_0 \quad \ldots\ldots\ldots (2)$$

Log C_0 was calculated from the regression equation of the logarithm of

the measured thiosulfate concentration in plasma on time, using in most of the cases the values obtained at 30 minutes and later (see Results).

The rate of disappearance of thiosulfate from plasma, k, was calculated as usual by the formula:

$$k = \frac{\log_e C_1 - \log_e C_2}{t_1 - t_2} \quad \ldots\ldots\ldots \text{(3a)}$$

or

$$k = 2.303 \cdot \frac{\log_{10} C_1 - \log_{10} C_2}{t_1 - t_2} \quad \ldots\ldots \text{(3b)}$$

where C_1 and C_2 are the plasma concentration of thiosulfate at time t_1 and t_2.

To increase the accuracy of the k-value, the average k for all points was calculated by substituting the regression coefficient b for $\frac{\log_{10} C_1 - \log_{10} C_2}{t_1 - t_2}$. The k-value is expressed in per cent per minute.

The apparent volume of distribution of thiosulfate at different times during the test in group III was calculated by the formule:

$$V_{TS}t = \frac{TS_{\text{inj}} - TS_{\text{excr.}}t}{C_t} \quad \ldots\ldots\ldots \text{(4)}$$

where $V_{TS}t$ is the apparent volume of distribution of thiosulfate in liters at time t, TS_{inj} is the amount of thiosulfate injected in mg, $TS_{\text{excr.}}t$ is the amount of thiosulfate excreted in the urine in mg over the period t_0 to t, and C_t is the plasma concentration of thiosulfate in mg per liter at time t.

Statistical methods.

The sampling errors of regression constants were calculated as described by MATHER (1949), the analysis of variance as described by DAVIES (1954). For the remaining statistical work the usual formulas were used (BONNIER and TEDIN 1940).

The transformation of a standard error (ε) in log-units to percentage of the mean was made according to the following:

ε per cent $= 100 \cdot \log_e 10 \cdot (\varepsilon)$ . . . . . (5)

(ε) = standard error in log units, and

ε per cent = standard error as percentage of the mean.

All $\pm$ values following the means in the text represent standard errors unless otherwise stated.

Results.

The equilibration time of thiosulfate in its space in man is usually given as 20 minutes. This could not be confirmed in the present study.

For each test the plasma thiosulfate concentration (C_t) was plotted against time (t) on a semilog paper. From inspection of these diagrams it could be readily seen that in most of the tests the 20 min. value lay above the straight line which could be drawn by inspection through the remaining

Table I.

Differences for the concentrations of thiosulfate in plasma at the 20th minute between the value found and the one calculated from the regression equation.

		Differences in $\sigma\,(y-Y)$											
		−						+					
		>10	10−5	5−3	3−2	2−1	1−0	0−1	1−2	2−3	3−5	5−10	>10
No. of tests....	42	0	1	0	0	2	0	2	3	4	15	8	7
Per cent of total number.	100	0	2.4	0	0	4.8	0	4.8	7.1	9.5	35.7	19.0	16.7

points. In five of the 43 tests in groups I and II (no. 1 b, 2 a, 5 b, 16 a, 20 b) this was also the case even for the 30 min. value. Therefore, in the calculation of the regression equation of log C_t on t, the 20 min. value in all tests and the 30 min. value in the above mentioned five tests were excluded.

The correctness of the impression obtained from inspection of the diagrams was verified as follows:

For each test the difference between log C_{20} as measured by analysis and log C_{20} as calculated from the regression equation was calculated, and this difference expressed in the individual $\sigma\,(y-Y)$ units. The results of these calculations in groups I and II are shown in Table I. It was found that only in 16.7 per cent of the tests was the found log C_{20} within the 2 sigma limits of the regression equations. In all the remaining, but one, the found log C_{20} was higher than the one expected from the regression equation.

The concentration of thiosulfate in plasma after equilibrium has been reached, is considered as a single exponential function of time. Therefore, in the calculation of the regression equations of log C_t on t a straight relationship between these two variables was assumed. To test whether this assumption was correct, the following calculations were performed:

For each test the difference between log C_t found by analysis and log C_t expected from the calculated regression equation were calculated and transformed in $\sigma\,(y-Y)$ units. The mean values of these differences at 30, 40, 50, 60, 70 and 80 min. for the tests in groups I II are shown in Table II. In the first part of the table (Table II a) the mean values for the individual tests are presented; in the second part (Table II b), the means presented were calculated from the mean of

Table II.

The difference between log C_t measured and log C_t calculated from the regression equations,

II a) from individual tests,

II b) from the mean of the differences in repeat tests.

Table II a.

Time min.	n	$\bar{x}$ [1]	$\varepsilon_{\bar{x}}$	P-value	$\bar{x}$ in per cent of C_t [2]
30′	38	+ 0.402	0.108	< 0.001	+ 0.52
40′	42	− 0.110	0.138	< 0.5	− 0.14
50′	42	− 0.393	0.153	< 0.02	− 0.51
60′	43	− 0.100	0.130	< 0.5	− 0.13
70′	41	+ 0.157	0.114	< 0.2	+ 0.20
80′	5	+ 0.604	0.387	< 0.8	+ 0.79

Table II b.

Time min.	n	$\bar{x}$ [1]	$\varepsilon_{\bar{x}}$	P-value	$\bar{x}$ in per cent of C_t [2]
30′	15	+ 0.458	0.118	< 0.01	+ 0.60
40′	19	− 0.089	0.159	> 0.5	− 0.11
50′	20	− 0.377	0.109	< 0.01	− 0.49
60′	20	− 0.155	0.147	> 0.2	− 0.20
70′	19	+ 0.362	0.092	< 0.001	+ 0.47
—	—	—	—	—	—

the differences in the repeat tests in subjects 1—20. If the relationship between log C_t and t was a linear one, it would be expected that the mean difference between found and calculated log C_t would not be statistically different from zero. It was found, however, that the differences at 30, 50 and 70 min. were probably significant to highly significant. These findings show that in the present study the total disappearance of thiosulfate from plasma was not a single exponential function of time.

The mean $\sigma(y-Y)$ in groups I and II was found to be $+0.00563$ corresponding to approximately ± 1.3 per cent of C_t. By aid of this value and equation 5 the mean differences in Table II were transformed in per cent of C_t. The differences observed varied between

[1] In $\sigma(y-Y)$ units.

[2] C_t denotes the plasma concentration of thiosulfate in mg per cent at time t.

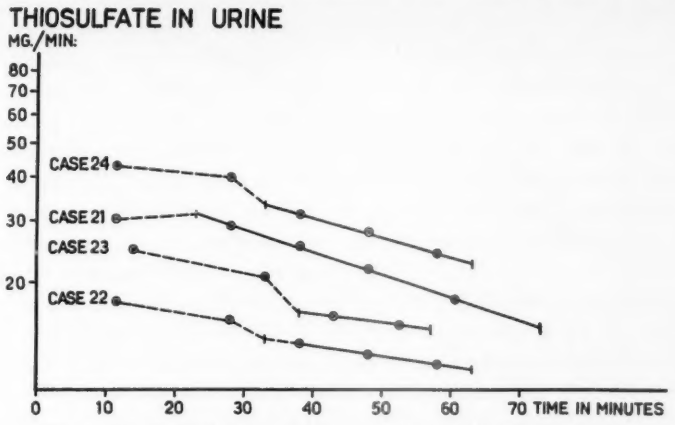

Fig. 1. Correlation between urinary excretion of thiosulfate and time.

-0.51 and $+0.79$ per cent of C_t. Thus, although log C_{TS} was not linear against t, the deviation from linearity was very small.

The excretion of thiosulfate in the urine was studied in cases 21—24 (group III) and the results are presented in Fig. 1. When the urinary excretion of thiosulfate (mg/min., mean for each collection period) was plotted against time (middle of each period) on semilog paper, a linear relationship was obtained after 40 minutes, disregarding urine delay time. This confirms the findings of NEWMAN *et al.* (1946).

The results of the measurements of *the apparent volume of distribution of thiosulfate at different times* during the tests in group III are presented in Table III. A progressive increase in $V_{TS}t$ was observed in all cases. Fig. 2 shows that the increase in $V_{TS}t$ was an exponential function of time.

The results of the measurements of the thiosulfate space in cases 1—20 are presented in Table IV[1]. The mean error of log V_{TS} as

[1] A part of these results have been presented in a previous report (IKKOS, 1955). In this report C_o was calculated after extrapolation by inspection. The differences of the V_{TS} values for a given test in the two reports are due to the different techniques used for the extrapolation.

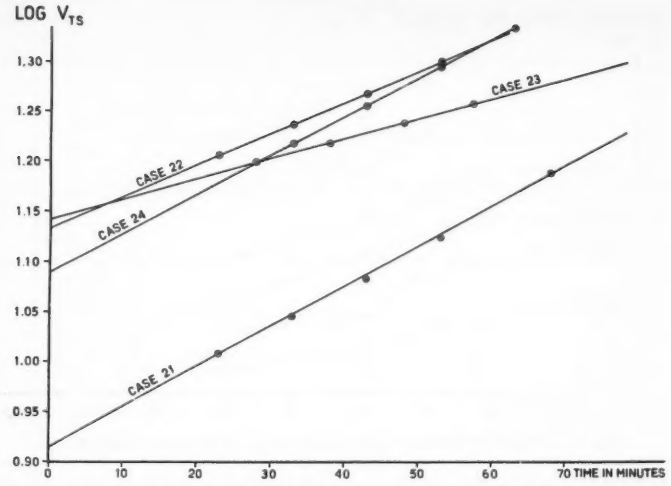

Fig. 2. Correlation between volume of distribution of thiosulfate (measured by difference) and time.

calculated from the regression analysis was 0.01086 for group I, 0.01093 for group II and 0.01088 for both groups. The values correspond to ± 2.50, ± 2.52 and ± 2.51 per cent of V_{TS}. The error of log V_{TS} was taken as identical with the error of log C_0 (equation 2).

The constancy of the apparent thiosulfate space was examined by performing repeat measurements in cases 1—19 with an interval of one to two weeks (Table IV). Probably significant to significant

Table III.

Volume of distribution of thiosulfate; calculated from the amount of thiosulfate not excreted in the urine and the concentration in plasma at a given time.

	Case 21		Case 22		Case 23		Case 24	
	time min.	V_{TS} liters	time min.	V_{TS} liters	time min.	V_{TS} liters	time min.	V_{TS} liters
1	23	10.2	23	16.0	28	15.8	33	16.5
2	33	11.1	33	17.2	38	16.5	43	18.0
3	44	12.1	43	18.5	48	17.3	53	19.7
4	53	13.3	53	20.0	57	18.1	63	21.7
5	68	15.4						

differences between the two measurements were found in two out of 13 cases in group I, and in two out of 7 cases in group II. The analysis of variance showed that the error of a single determination as calculated from repeat measurements was, for log V_{TS}, 0.01225 in group I, and 0.03647 in group II, corresponding to 2.8 and 8.4 per cent of the mean V_{TS}. The difference between the two groups was significant ($P<0.01$). Further, the analysis of variance showed a significant difference (-0.018) between the first and second measurement in group I ($P<0.01$) but not in group II ($P>0.2$).

In case 20 in which 5 measurements of thiosulfate space were made over a period of one year (body weight constant within ± 1 kg.), no statistical differences between the repeated measurements were found.

The rate of disappearance of thiosulfate from plasma is presented in Table IV. The mean k-value was found to be 1.47 ± 0.05 in group I, and 1.27 ± 0.17 in group II, the difference being not significant. Probably significant to significant differences between the repeat k-values were found in one of the 13 cases of group I, and in 4 of the 7 cases of group II.

The analysis of variance showed that the mean difference between repeat k-values was larger in group II than in group I ($P<0.001$). A probably significant increase of the k-value in the second measurement was found in group I ($P<0.05$) but not in group II ($P>0.2$).

No significant correlation was found between the difference of two measurements of V_{TS} in the same subject and the difference of the corresponding k-values ($P>0.05$).

Discussion.

The discrepancy between the present results and those of CARDOZO and EDELMAN (1952) as regards the time of equilibration of thiosulfate in the body cannot be explained.

The finding that the plasma concentration of thiosulfate (C_t) after equilibrium was not a single exponential function of time (Table III) is not in agreement with previous reports (GILMAN *et al.* 1946, NEWMAN *et al.* 1946, SCHWARTZ 1950, CARDOZO and EDELMAN 1952, FRIIS-HANSEN 1954). These authors reported that as judged by inspection of diagrams log C_t was linear against t after equilibrium of thiosulfate. The changes of C_t can, theoretically, be considered as depending on the urinary loss of thiosulfate, and the transfer of

Table IV.

Repeat measurements of the volume of distribution of thiosulfate in 20 subjects

Case[3] No.	Interval between repeat tests, days	Thio-sulfate injected g	Regression equation of log C_t on t [1]						V_{TS}		k-value
			n	$\bar{x}$	$-b$	ε_b	log C_o		log V_{TS}	in liters	
							log C_o	ε log C_o			
1 a	3	10.0	5	50.0	0.005540	0.000574	2.96167	0.00908	1.03833	10.92	1.28
b		10.0	4	64.6	0.005717	0.000139	2.95654	0.00914	1.04346	11.05	1.32
2 a	6	10.0	6	55.3	0.006117	0.000146	2.93872	0.00840	1.06128	11.52	1.41
b		10.0	5	60.0	0.006044	0.000114	2.91268	0.00707	1.08732	12.23	1.39
3a	6	10.0	5	50.8	0.006423	0.000353	2.93332	0.01850	1.06668	11.66	1.46
b		10.0	6	55.1	0.006372	0.000187	2.95382	0.00330	1.04618	11.12	1.47
4 a	5	10.0	5	49.7	0.008078	0.000206	3.00320	0.01076	0.99680	9.93	1.86
b		7.5	5	50.0	0.008189	0.000289	2.82127	0.01443	1.05379 [2] *	11.32 [2] *	1.89
5 a	4	10.0	4	55.8	0.005848	0.000273	2.86154	0.01552	1.13846	13.76	1.35
b		10.0	5	50.0	0.006675 [2] *	0.000141	2.83567	0.00707	1.16433	14.60	1.54
6 a	4	10.0	5	49.7	0.005428	0.000183	2.85764	0.00940	1.14236	13.88	1.25
b		10.0	5	50.0	0.005191	0.000129	2.83678	0.00646	1.16322	14.56	1.20
7 a	7	7.5	5	49.5	0.006303	0.000129	2.85842	0.00664	1.01664	10.39	1.45
b		7.5	5	50.0	0.006861	0.000216	2.86645	0.01080	1.00976	10.23	1.58
8 a	4	10.0	5	49.8	0.005502	0.000099	2.83078	0.00513	1.16922	14.77	1.27
b		10.0	5	50.0	0.006036	0.000494	2.82980	0.02471	1.17020	14.80	1.39
9 a	4	7.5	5	50.0	0.006532	0.000153	2.87472	0.00793	1.00034	10.01	1.50
b		7.5	5	50.0	0.006720	0.000018	2.89549	0.00095	0.97057 [2] *	9.54 [2] *	1.55
10 a	9	7.5	5	50.0	0.006635	0.000115	2.90867	0.00600	0.96693	9.27	1.53
b		7.5	5	50.0	0.006708	0.000216	2.88514	0.01123	0.98992	9.77	1.54
11 a	10	7.5	5	50.0	0.005642	0.000032	2.86002	0.00158	1.01504	10.35	1.30
b		7.5	5	50.8	0.005862	0.000175	2.85456	0.00290	1.02050	10.48	1.35
12 a	13	10.0	3	50.0	0.006423	0.000316	2.91166	0.01602	1.08834	12.26	1.48
b		7.5	5	50.0	0.006962	0.000259	2.78414	0.01345	1.09092	12.33	1.60
13 a	9	10.0	5	50.0	0.004268	0.000115	2.53786	0.00600	1.46214	28.98	0.98

12 a	13	10.0	3	50.0	0.006423	0.000316	2.91166	0.01602	1.08834	12.26	1.48
b		7.5	5	50.0	0.006962	0.000259	2.78414	0.01345	1.09092	12.33	1.60
13 a	9	10.0	5	50.0	0.004268	0.000115	2.53786	0.00600	1.46214	28.98	0.98
b		10.0	5	50.1	0.003501 [2] **	0.000181	2.46108 [2] ***	0.00941	1.53892 [2] ***	34.59 [2] ***	0.81
14 a	8	10.0	5	49.4	0.007215	0.000317	2.65546	0.01627	1.34454	22.11	1.66
b		10.0	5	50.0	0.009101 [2] **	0.000216	2.69849	0.01123	1.30151	20.02	2.10
15 a	6	10.0	5	49.4	0.006634	0.000259	2.72953	0.01034	1.27047	18.64	1.53
b		10.0	5	49.6	0.006745	0.000231	2.65628	0.01195	1.34372 [2] **	22.07 [2] **	1.55
16 a	7	10.0	5	50.0	0.006224	0.000187	2.81501	0.00972	1.18499	15.31	1.43
b		10.0	5	50.0	0.005135 [2] **	0.000142	2.79991	0.00740	1.20009	15.85	1.19
17 a	8	5.0	5	50.0	0.003514	0.000061	2.63224	0.00315	1.06673	11.66	0.81
b		5.0	5	50.0	0.003735	0.000225	2.65123	0.01170	1.04774	11.16	0.86
18 a	8	7.5	5	50.0	0.006846	0.000401	2.88133	0.02085	0.99373	9.86	1.58
b		7.5	5	50.0	0.007912 [2] *	0.000158	2.93227	0.00822	0.94279	8.77	1.82
19 a	17	10.0	5	51.2	0.003376	0.000187	2.87498	0.00998	1.12502	13.34	0.78
b		10.0	7	70.1	0.003024	0.000094	2.87862	0.00305	1.12138	13.22	0.70
	Date										
20 a	13.1.54	7.5	4	49.4	0.006943	0.000122	2.81442	0.00624	1.06064	11.50	1.60
b	21.1.54	7.5	5	49.6	0.007462	0.000162	2.82618	0.00836	1.04888	11.09	1.72
c	28.1.54	7.5	5	49.6	0.006456	0.000162	2.79270	0.00806	1.08236	12.09	1.49
d	3.2.54	7.5	5	50.2	0.007435	0.000231	2.82416	0.01204	1.05090	11.24	1.71
e	18.1.55	7.5	4	45.0	0.007386	0.000339	2.83302	0.01572	1.04204	11.02	1.70

[1] C_t denotes concentration of thiosulfate in plasma (in mg per liter) at time t (in minutes).

[2] *, **, *** denote significance of difference between two tests on the same subject; the absence of a star denotes that the difference found was not significant.

[3] a–e denote repeat measurements in chronological order.

thiosulfate across the capillary membranes between plasma and the remaining volume of distribution of thiosulfate. Assuming that 1) the urinary excretion of the substance is proportional to C_t, 2) that the transfer across the capillaries is proportional to the difference between the concentrations, and 3) that extrarenal losses of thiosulfate from the interstitial fluid are proportional to the thiosulfate concentration in this compartment, then:

$$\begin{aligned} dx &= C_1 (y-z) \cdot dt - C_2 \cdot x \cdot dt \\ dy &= C_1 (x-y) \cdot dt - C_3 \cdot y \cdot dt \end{aligned} \quad \ldots\ldots (6)$$

where x is the concentration of thiosulfate in plasma,

y is the concentration of thiosulfate in V_{TS} minus plasma, and

dx and dy changes of x and y during a short element of time dt.

From equation (6) it follows that

$$C_t = C_1 e^{-\lambda_1 t} + C_2 e^{-\lambda_2 t} \quad \ldots\ldots\ldots (7)$$

where C_1 and C_2 are the concentrations obtained by extrapolation of the two components to time zero, and λ_1 and λ_2 are the slopes of the two components.

Elimination of assumption (3) would not change the general expression of equation (7).

Equation (7) is similar to the usual equation (DOMINGUEZ 1950) for the description of the kinetics of substances in the body. Moreover, it is similar to the equation of CARDOZO and EDELMAN (1952) used for the description of the thiosulfate changes in plasma during the initial period before equilibrium of the substance.

The changes in C_t would be a single exponential function of time after equilibrium only if one of the factors in equation (7) disappeared. This, however, seems improbable when renal elimination of thiosulfate is present. The urinary loss can be expected to induce a concentration gradient across the capillary membranes, which gradient causes the transfer of thiosulfate from interstitial fluid for excretion. The theoretical possibility for the disappearance of the capillary gradient exists when the decrease in C_t in plasma from the urinary excretion is identical with the decrease of C_t in interstitial fluid from the extrarenal losses of the substance (metabolization, penetration to new fluid compartments). This, however, seems improbable in ordinary cases, in which most of the thiosulfate is excreted in the urine (GILMAN *et al.* 1946, NEWMAN *et al.* 1946, CARDOZO and EDELMAN 1952).

$V_{TS}t$ was found to increase progressively (Table III). This shows that thiosulfate disappears from the body by other routes than the urine, as previously demonstrated by others (GILMAN *et al.* 1946, NEWMAN *et al.* 1946, CARDOZO and EDELMAN 1952). The logarithmic increase in $V_{TS}t$ with t (Fig. 2) supports the view of a double exponential function of time on Ct. When equation (4) is solved with respect to Ct with this finding in mind an equation of the same type as equation (7) is obtained.

The urinary excretion of thiosulfate was found to be an exponential function of time (Fig. 1). This finding has been reported earlier (NEWMAN *et al.* 1946), and supports assumption (1). As to assumption (3), support is provided by the logarithmic decrease of C_t with t in nephrectomized animals (GILMAN *et al.* 1946, BOLING and WILSON 1953, SWAN 1954).

The difference between the plasma and interstitial fluid concentration of thiosulfate, large at the beginning of the test, decreases as thiosulfate reaches equilibrium. The decrease in this difference can explain the observation that the changes in C_t after equilibrium approach a single exponential function of time, and that the differences between the actual line for log Ct and the calculated straight one is small (Table II). For practical purposes, therefore, the relation between log C_t and t after equilibrium can be taken as a linear one.

The assumption of a straight relationship between log C_t and t in the calculation of C_0 increases the error of V_{TS} determination. Despite this, the error found for a single measurement of the apparent V_{TS} ($\pm 2.52\,\%$) is small, and can be favorably compared with the errors of other methods used for the measurement of the extracellular fluid.

It has been shown that variations in the time of injection of the thiosulfate can influence its apparent volume of distribution (RAISZ *et al.* 1953). This source of variation of the apparent V_{TS} was eliminated in the present study by employing a constant time of injection.

The reproducibility of the apparent V_{TS} in group I (± 2.8 per cent) was good, and in agreement with the values presented by CARDOZO and EDELMAN (1952). The larger variation between repeat measurements in group II (± 8.4 per cent) might be due to a greater instability of the extracellular fluid volume or to the larger variability of the k-value between repeat measurements in this group (BOLING and WILSON 1953, IKKOS *et al.* 1956).

The systematic difference between the repeat measurements in cases 1—12 is difficult to explain. It should be mentioned that 10 of the first measurements of apparent V_{TS} against 2 of the second measurements were performed in connection with inulin space determinations (IKKOS 1955). During these measurements, thiosulfate was injected between 2—4 p.m., while in the remaining tests the injection was given in the morning. The prolonged fasting and recumbency of the patients might have induced changes in the extracellular fluid volume. Another possibility was that the difference in the state of the patients influenced the apparent V_{TS} by changes in the k-value (BOLING and WILSON 1953, IKKOS *et al.* 1956). This explanation seems probable as a probably significant difference between the repeat k-measurements was obtained. However, the explanation cannot be considered as proved in the present material as no significant correlation could be demonstrated between the differences of repeat measurements of V_{TS} and the differences of the corresponding k-values.

Summary.

The measurement of the apparent volume of distribution of thiosulfate after a single injection was examined in 43 tests on 13 normal subjects and 11 patients with different disorders.

The time of equilibration of thiosulfate was found in most of the tests to lie between 20 and 30 minutes after the beginning of the injection. The changes in the concentration of thiosulfate in plasma could be described as the sum of two exponential functions with respect to time.

The overall error of a single measurement of the thiosulfate space was ± 2.50 per cent.

The apparent volume of distribution of thiosulfate was reproducible with an accuracy of ± 2.8 per cent in normal subjects and ± 8.4 per cent in pathological cases.

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Measurement of the Extracellular Fluid Volume by Thiosulfate.

II. The relation between the apparent volume of distribution of thiosulfate and the volume of distribution of inulin.

By

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Simultaneous measurements of the volumes of distribution of inulin (V_{IN}) and thiosulfate (V_{TS}) in human subjects showed a good agreement between these volumes, the mean V_{IN} to V_{TS} ratio being 0.95. In some cases, however, there were rather large discrepancies between V_{IN} and V_{TS}, the ratio of the former to the latter varying between 0.74 and 1.36 (IKKOS 1955).

The aim of the present work was to examine further the relation between the volumes of distribution of thiosulfate and inulin in human subjects.

Material and Methods.

The material comprises 38 subjects, 20 males and 18 females, ranging in age between 20 and 54 years. Group I (cases 1—24) comprises healthy subjects and patients with minor disorders, in which changes in the metabolism or distribution of water in the body were not obvious or probable. Group II (cases 25—38) comprises 12 patients with acromegaly, one with hypokalemia, and one with Cushing's syndrome.

The apparent volume of distribution of thiosulfate was measured by the single injection technique (CARDOZO and EDELMAN 1952), and the volume of distribution of inulin by the constant infusion-urine collection technique

Table I a.

The volumes of distribution of thiosulfate and inulin in 24 healthy adults.

Case no.	Sex	Age years	Interval days[1]	V_{TS} liters	V_{TS} k-value	V_{IN} liters
1 a.....	M	22	0	10.92	1.28	9.1
b.....			3	11.05	1.32	
2 a.....	M	23	0	12.23	1.39	12.0
b.....			6	11.52	1.41	
3 a.....	M	56	0	11.66	1.46	11.9
b.....			6	11.12	1.47	
4 a.....	F	20	0	9.93	1.86	10.1
b.....			5	11.32	1.89	
5 a.....	M	23	0	14.60	1.54	12.5
			4	13.76	1.35	
6 a.....	M	44	0	13.88	1.25	11.9
b.....			4	14.56	1.20	
7 a.....	F	52	0	10.39	1.45	10.6
b.....			7	10.23	1.58	
8 a.....	M	23	0	14.77	1.27	12.6
b.....			4	14.80	1.39	
9 a.....	F	27	0	10.01	1.50	9.1
b.....			4	9.54	1.55	
10 a.....	F	24	0	9.27	1.53	8.8
b.....			9	9.77	1.54	
11.....	F	24	0	8.11	1.37	6.4
12.....	F	26	0	10.63	1.18	8.5
13.....	F	52	0	8.51	1.68	9.3
14.....	M	23	18	11.09	1.67	10.2
15.....	F	26	2	10.78	1.98	12.4
16.....	F	23	5	10.87	1.59	10.4
17.....	F	24	1	11.79	1.56	11.6
18.....	M	54	8	11.26	1.57	11.1
19.....	M	35	6	12.46	1.65	12.5
20.....	M	26	1	12.70	1.59	12.4
21.....	M	21	7	11.69	1.59	12.7
22.....	M	22	16	13.46	1.63	12.0
23.....	M	38	2	9.31	1.66	8.3
24.....	M	30	1	13.02	1.41	11.7

[1] Interval between V_{TS} and V_{IN} measurements.

of GAUDINO *et al.* (1948). More details of the measurements of these two spaces have been presented elsewhere (IKKOS 1955, 1956).

In cases 1—13 and 25—32, thiosulfate and inulin spaces were measured simultaneously, as previously described (IKKOS 1955). The thiosulfate space was measured once more a few days earlier or later in cases 1—10 and 25—27. In the remaining cases (14—24, 28 b and 33—38) the inulin and thiosulfate spaces were measured on separate days with an interval of 1 to 20 days. In cases 27, 28, and 33 the measurements of the inulin and thiosulfate spaces were repeated within one year.

Table I b.

The volumes of distribution of thiosulfate and inulin in 14 pathological cases.

Case no.	Sex	Age years	Diagnosis	Interval days[1]	V_{TS} liters	V_{TS} k-value	V_{IN} liters
25 a....	M	42	Acromegaly	0	34.59	0.81	26.7
b....				9	28.98	0.98	
26 a....	M	61	Acromegaly	0	18.64	1.53	19.9
b....				6	22.07	1.55	
27 a....	M	23	Acromegaly	0	22.11	1.66	20.5
b....				8	20.02	2.10	20.3
c....				0	15.11	3.09	
28 a....	F	50	Acromegaly	0	16.74	1.42	14.5
29.....	M	?	Acromegaly	0	18.40	1.89	17.7
30.....	F	35	Acromegaly	0	10.43	1.73	10.8
31.....	M	22	Acromegaly	0	13.42	2.52	19.1
32.....	F	27	Hypokalemia	0	7.74	0.76	5.8
28 b....	F	51	Acromegaly	15	17.38	1.37	16.5
33 a....	F	27	Acromegaly	7	16.51	1.45	15.7
b....		26		6	14.31	1.44	13.8
34.....	F	35	Acromegaly	20	13.26	1.96	12.5
35.....	F	42	Acromegaly	10	12.04	1.62	10.9
36.....	M	32	Acromegaly	10	20.92	2.08	24.4
37.....	F	36	Acromegaly	1	16.63	2.57	22.6
38.....	F	28	Cushing's syndrome	3	8.82	1.74	8.7

[1] Interval between V_{TS} and V_{IN} measurements.

Statistical methods.

The analysis of covariance was performed according to SNEDECOR (1948). The sampling errors of the regression constants were calculated according to MATHER (1949). The remaining calculations were performed by the usual methods (BONNIER and TEDIN 1940).

Results.

The results of the measurements of the thiosulfate and inulin spaces are presented in Table I. In the same table is also presented the rate of disappearance of thiosulfate from plasma (k-value) in the different tests.

The ratio V_{IN} to V_{TS} was found to be 0.94 ± 0.02[2] in group I, and

[2] All $\pm$ values after the means represent standard errors.

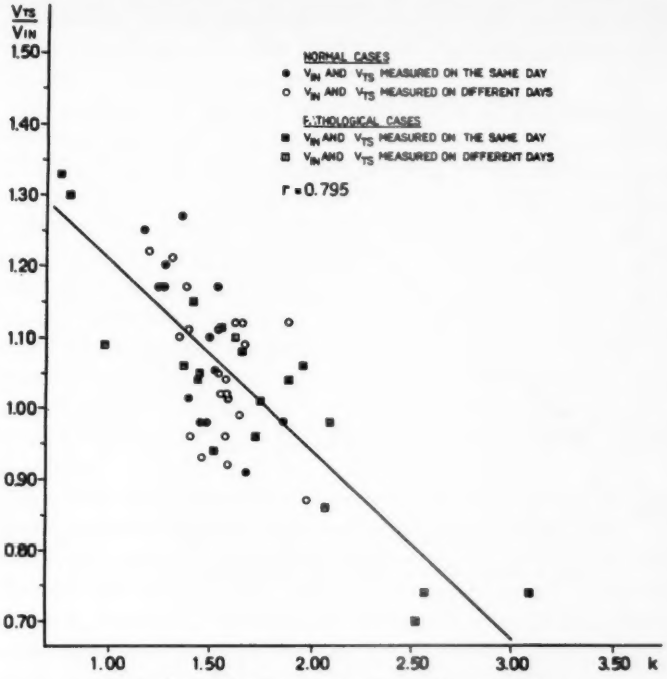

Fig. 1. Correlation between the V_{TS} to V_{IN}-ratio and k.

1.01 ± 0.04 in group II. The difference between these values was not significant. The mean V_{IN} to V_{TS} ratio for all tests was 0.97 ± 0.02. The coefficient of variation was 9.8, 18.0 and 13.9 per cent for groups I, II, and I + II, respectively.

In Fig. 1, the V_{TS} to V_{IN} ratio was plotted against k. Under the assumption of a straight relationship, a significant negative correlation ($P < 0.001$) was found between the two variables.

In Fig. 2 V_{IN} was plotted against V_{TS}. A highly significant correlation was found between the two spaces ($P < 0.001$). The regression equations of V_{IN} on V_{TS} in the present material are shown in Table II. The regression equations obtained for groups I and II were highly significant ($P < 0.001$). The regression line was probably not passing through the origin in group I ($P < 0.05$); it did pass through the

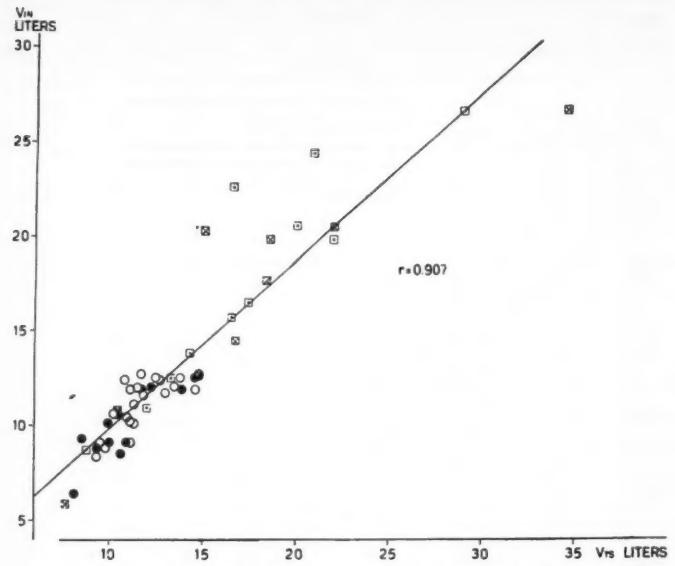

Fig. 2. The relation between V_{IN} and V_{TS} (signs as in Fig. 1).

origin in group II. The error of estimate of V_{IN} from V_{TS} (in per cent of the mean V_{IN}) was found to be 9.2 and 17.1 in groups I and II, respectively. The analysis of covariance showed a significant difference between the two regressions ($P < 0.01$), but no significant difference between the two regression coefficients.

Table

Regression equations

	n	Variables			
		V_{TS} $\bar{x} \pm \varepsilon_{\bar{x}}$ in liters	V_{IN} $\bar{y} \pm \varepsilon_{\bar{y}}$ in liters	$\sigma\bar{y}$	
				σ_{tot}	per cent of $\bar{y}$
Group I.....	34	11.51 ± 0.31	10.79 ± 0.28	1.64	15.20
Group II.....	20	17.40 ± 1.44	17.38 ± 1.30	5.79	33.31

The mean k-value was found to be 1.53 ± 0.04 in group I and 1.75 ± 0.14 in group II. The variation of k was significantly larger in group II than in group I ($P < 0.001$).

The influence of the rate of disappearance of thiosulfate from plasma (k-value) on the correlation between V_{IN} and V_{TS} is shown in Tables III and IV. When the effect of k was eliminated, the correlation became closer, as shown by the increase in the correlation coefficient. The multiple regression equations of V_{IN} och V_{TS} and k are presented in Table IV. The regression coefficients b_1 and b_2 were both significant to highly significant for all equations. The fact that the regression coefficient for k (b_2) was significant, confirms the results of the partial correlation analysis, and shows that the influence of k on the correlation of V_{IN} and V_{TS} was significant. The analysis of covariance showed no significant difference between the regression equations for groups I and II or their regression coefficients ($P > 0.05$).

The error of estimate of V_{IN} from V_{TS} and k (in per cent of the mean V_{IN}) was found to be 7.8, 8.8 and 8.9 in groups I, II, and I + II respectively. Thus, in group II the error of estimate of V_{IN} decreased when the effect of k was taken into account. This decrease is illustrated in Fig. 3, where V_{IN} corrected for variations in k (EZEKIEL 1950) is plotted against V_{TS}. It can be seen that the spreading of the points around the regression line in Fig. 3 is smaller than in Fig. 2.

The results were divided into two groups with respect to whether the V_{IN} and V_{TS} measurements were performed simultaneously or not. The covariance analysis showed no statistical difference between the regression equations of V_{IN} on V_{TS} and k in these two groups ($P > 0.05$).

II.

of V_{IN} on V_{TS}.

Regression						Coeff. of correlation r
$a \pm \varepsilon_a$	$b \pm \varepsilon_b$	$\sigma(y-Y)$		Significance P	Significance of diff. between zero and a P	
			per cent of $\bar{y}$			
2.41 ± 1.10	0.73 ± 0.09	0.99	9.2	< 0.001	< 0.05	0.806
3.85 ± 1.95	0.78 ± 0.11	2.97	17.1	< 0.001	< 0.1	0.867

Table III.

The influence of k on the correlation of V_{IN} to V_{TS}.

	n	Coeffic. of correlation	
		r_{12}	$r_{12.3}$
Group I.....	34	0.806	0.867
Group II.....	20	0.867	0.964
All tests.....	54	0.907	0.970

r_{12} denotes the coeff. of correlation between V_{IN} (x_1) and V_{TS} (x_2).
$r_{12.3}$ denotes the coeff. of correlation between V_{IN} (x_1) and V_{TS} (x_2) after elimination of the effect of k (x_3).

Discussion.

The measurement of the volume of distribution of thiosulfate by the technique used in the present study is based on the assumption that the concentration of thiosulfate in plasma at zero time (C_0) as obtained by extrapolation, equals the one which would be found, if the substance were instantly equilibrated in its volume of distribution (CARDOZO and EDELMAN 1952). Under such an assumption one might expect that the C_0 found would depend only on the amount of thiosulfate injected and its volume of distribution. However, it has been shown in dogs that C_0 is also influenced by changes in the rate of disappearance of thiosulfate from plasma, a decrease in k being

Table IV.

Regression equations of V_{TS}

	n	Variables					
		V_{TS} $\bar{x}_1 \pm \varepsilon_{\bar{x}_1}$ in liters	k $\bar{x}_2 \pm \varepsilon_{\bar{x}_2}$ in per cent per minute	V_{IN} $\bar{y} \pm \varepsilon_{\bar{y}}$ in liters	$\sigma\bar{y}$ σ_{tot}	$\sigma\bar{y}$ per cent of $\bar{y}$	$a \pm \varepsilon_a$
Group I..	34	11.51 ± 0.31	1.51 ± 0.03	10.79 ± 0.28	1.64	15.20	− 3.33 ± 1.62
Group II.	20	17.40 ± 1.44	1.71 ± 0.13	17.38 ± 1.30	5.79	33.31	− 5.78 ± 1.50
All tests..	54	13.69 ± 0.68	1.59 ± 0.05	13.25 ± 0.67	4.90	37.04	− 6.31 ± 0.81

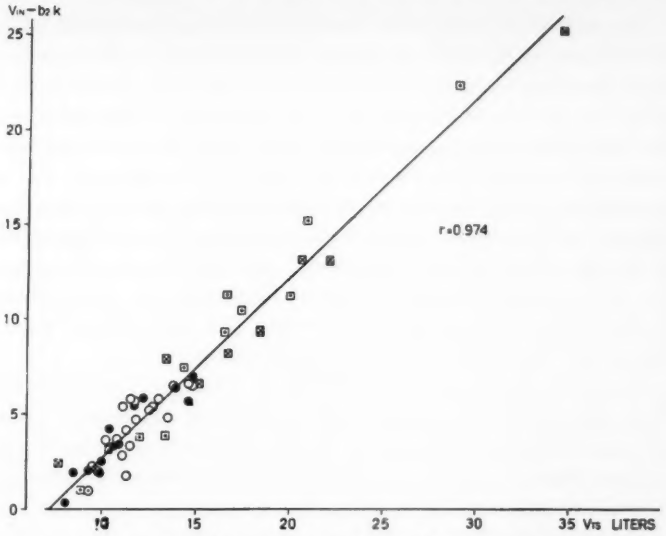

Fig. 3. The relation between V_{IN}, and V_{TS} and k (signs as in Fig. 1).

followed by a decrease in C_0 (BOLING and WILSON 1953). Therefore, the found C_0 cannot be considered as identical with the theoretical one and, consequently, the apparent V_{TS} cannot be taken as equal to the real V_{TS}.

Table IV.

of V_{TS} on V_{IN} and k.

Regression							
		$\sigma(y-Y)$		Significance (P)			
$b_1 \pm \varepsilon_{b_1}$	$b_2 \pm \varepsilon_{b_2}$		per cent of $\bar{y}$	of b_1	of b_2	of regression	of diff. between zero and a
0.84 ± 0.09	2.98 ± 0.84	0.84	7.83	<0.001	<0.01	<0.001	<0.05
0.89 ± 0.06	4.43 ± 0.63	1.52	8.76	<0.001	<0.001	<0.001	<0.01
0.92 ± 0.03	4.42 ± 0.42	1.17	8.85	<0.001	<0.001	<0.001	<0.001

The magnitude of the real V_{TS} can only be approximated.[1] The measurement of V_{TS} after elimination of the urinary loss of thiosulfate seems at present the best available approximation. Under such a condition the rate of disappearance of thiosulfate is very small and the measurement of V_{TS} approaches ideal conditions. In nephrectomized dogs apparent V_{TS} was larger than V_{IN} or apparent V_{TS} of intact animals, the values found approaching those for chloride space (BOLING and WILSON 1953, SWAN *et al.* 1954). In direct measurements of the thiosulfate space in tissues, V_{TS} was found to be larger than the volume of distribution of inulin (NICHOLS *et al.* 1953). These results suggest that the real V_{TS} is larger than the apparent V_{TS} as well as the functional extracellular fluid volume.

The present finding of an increase in the V_{TS} to V_{IN} ratio above 1 for low values of k (see above) supports this view. Moreover — under the assumption that real V_{TS} and V_{IN} are proportional — this finding suggests that an increase in k induces an increase in the difference between the real and the apparent V_{TS}.

The differences between the two groups as regards the variation of the V_{IN} to V_{TS} ratio, and the decrease in the error of estimate when k was taken into account, can be explained by the larger variability of the k-values in group II.

The disappearance of the difference between the two groups in the regression equation of V_{IN} on V_{TS} (Table II) when the rate of disappearance was taken into account (Table III) suggests that the difference found was due to differences in k.

The close agreement between apparent V_{TS} and V_{IN} ($V_{TS} : V_{IN}$ ratio 0.97) cannot be taken as showing that thiosulfate and inulin are distributed in the same finite fluid compartment, as previously suggested (TURNER and LEVITT 1953, IKKOS 1955).

Despite the fact that the apparent V_{TS} cannot be considered as representing the volume of any given fluid compartment, its measurement offers a simple way of calculating the functional extracellular fluid volume, the latter one taken as identical with V_{IN}. From the multiple regression equations in Table IV it can be seen that V_{IN} can be calculated from apparent V_{TS} and k with an accuracy of approximately $\pm$ 8 per cent.

[1] An equation for the calculation of the volume of distribution of metabolizable substances is presented by DOMINGUEZ (1950).

As the multiple regression equations for groups I and II separately did not differ significantly, the regression equation calculated from all tests is suggested for use in a material similar to the present one. This equation is

$$V_{IN} = -6.31 + 0.92\,V_{TS} + 4.42\,k$$

where V_{IN} and V_{TS} are expressed in liters and k in per cent per minute.

The principal advantage of such a calculation of the functional extracellular fluid over its measurement by other methods is the rapidity and the simplicity of the thiosulfate method.

Summary.

The volumes of distribution of inulin and thiosulfate were compared in 24 healthy adults and 14 patients. The apparent volume of distribution of thiosulfate (V_{TS}) was found to be of the same magnitude as that of inulin (V_{IN}), the mean $V_{TS} : V_{IN}$ ratio being 1.03 ± 0.02.

The discrepancies between apparent V_{TS} and V_{IN} were rather large, the $V_{TS} : V_{IN}$ ratio ranging between 0.70 and 1.33 with a coefficient of variation of 13.9 per cent. These discrepancies could be in part accounted for by the variations of the rate of disappearance of thiosulfate from plasma.

An equation is presented for the calculation of the volume of distribution of inulin from the apparent volume of distribution of thiosulfate.

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Growth Failure Caused by Mild Heat Treatment of the Diet.

By

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Received 30 August 1955.

In connection with some work on the possible carcinogenic effect of a mildly heated died it was found that even a slight browning caused a cessation of the growth of rats used as test animals. The diet, which was supplemented with vitamins, was made up to correspond approximately to the average food of the Finnish people. The following experiments were carried out to find an explanation and a possible remedy for the growth failure.

Experimental and results.

The Wistar rats used in these experiments were weaned at the age of 21 days and thereafter fed the diets and water ad libitum. They were housed in individual cages under temperature controlled conditions. The experimental diet was composed as follows:

Diet no. 123

Whole wheat meal	2000 gm	or 35.5 %
Potatoes	2000 »	» 35.5 %
Whole milk powder	700 »	» 12.5 %
Minced beef	450 »	» 8.0 %
Sucrose	250 »	» 4.4 %
Lard	200 »	» 3.6 %
Sodium chloride	25 »	» 0.5 %

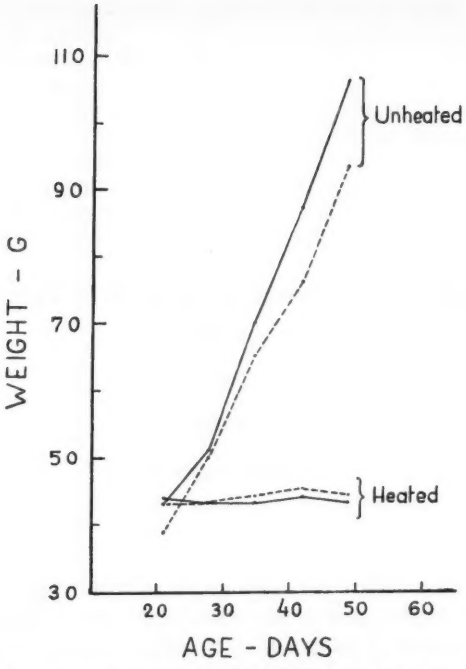

Fig. 1. Growth curves of rats on the unheated and the heated diet. Each curve represents the average of ten animals.

Most of the water contained in the diet was evaporated in an oven at 50°—60° C. In this procedure the mixture lost about one third of its weight. It was then finely ground and a vitamin mixture was added to give the following amounts per kilogram of the diet: thiamin 2 mg, riboflavin 4 mg, pyridoxin 2 mg, calcium panthothenate 20 mg, nicotinic acid 25 mg, choline chloride 1000 mg, inositol 200 mg, biotin 0.1 mg, folic acid 2 mg, p-aminobenzoic acid 5 mg, and 2-methyl-1, 4-naphthoquinone 5 mg. This diet (no. 123 A) was fed to the control animals.

The heated diet (no. 123 B) was prepared from the above mixture by keeping it in layers $^1/_2$ inch deep in an oven at 140° until it developed a slightly brown colour and a palatable taste, which took 30 to 40 minutes. During the heating the layers were turned over every tenth minute. Before use the heated diet was supplemented with the vitamin mixture as in case of the unheated diet. In addition the rats on the both diets received 1 drop of cod liver oil per week.

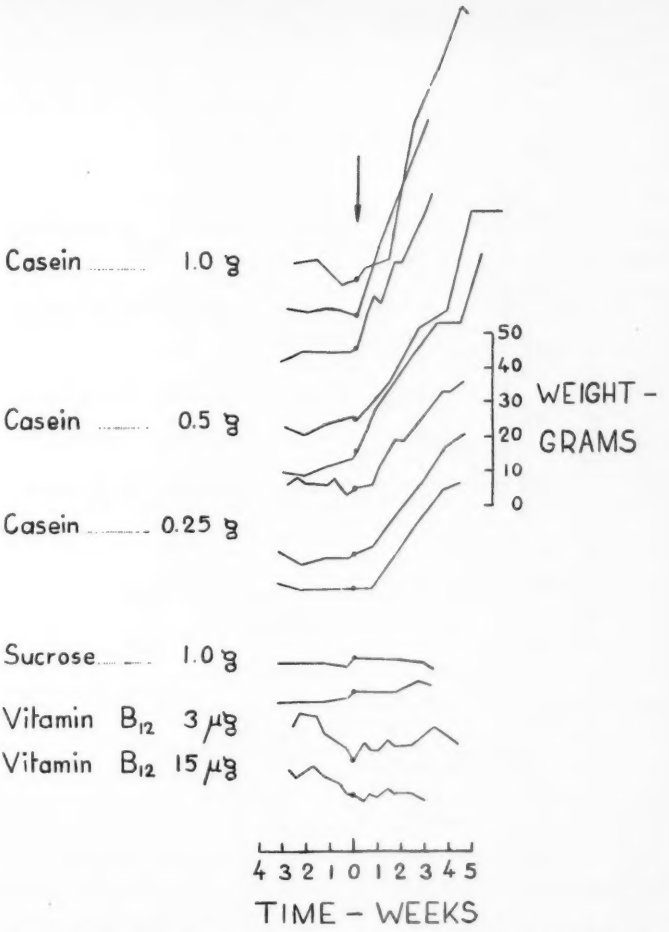

Fig. 2. Individual growth curves of rats on the heated diet supplemented with casein, sucrose or vitamin B_{12}. The figures on the left give the daily dose per rat. The arrow shows the time when the supplementation was started.

The growth-promoting qualities of the unheated and the heated diets were quite different. While the former (123 A) enabled a good growth, the weight of the animals on the latter (123 B) remained

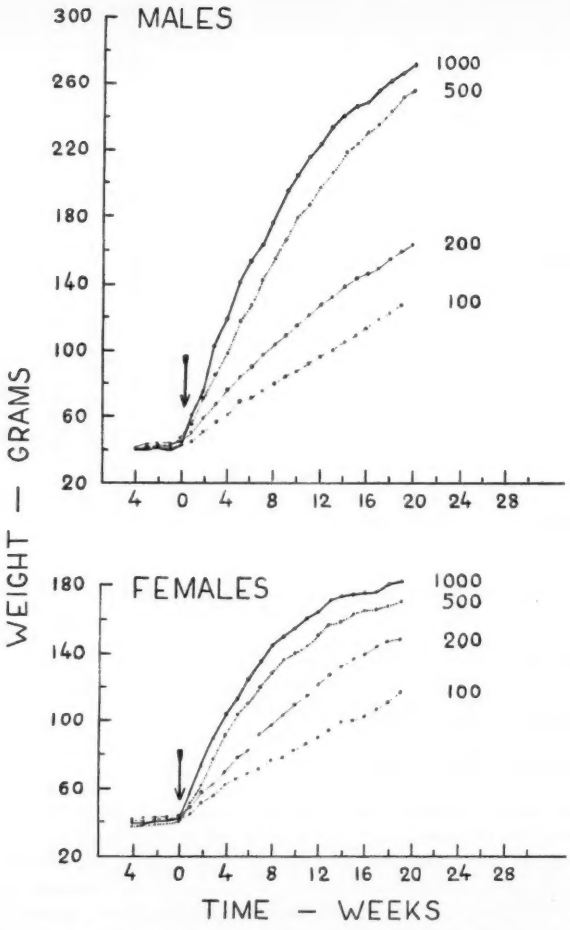

Fig. 3. Growth. of rats on the heated diet supplemented with casein. Each curve represents the average of 6—10 animals. The figures on the right give the daily dose per rat in milligrams. The arrows denote the start of the supplementation.

nearly constant. The latter animals remained at their weaning weight in some cases for 16 weeks. Nevertheless, they seemed quite healthy with bright eyes, smooth hair and a lively temperament. The difference

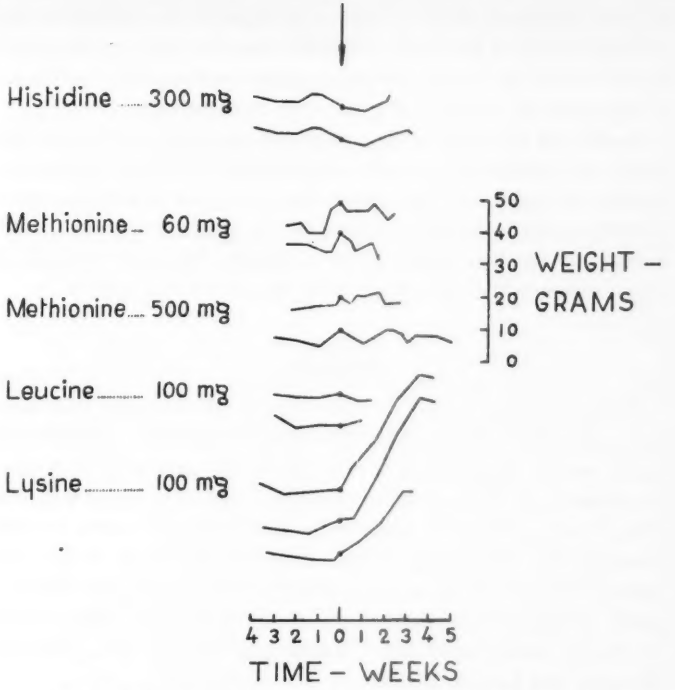

Fig. 4. Individual growth curves of rats on the heated diet supplemented with different amino acids.
The figures on the left give the daily dose per rat.
The arrow denotes the start of the supplementation.

between the growth rates of rats on the unheated and the heated diet is shown in Figure 1.

The possible curative effect of casein, sucrose, and vitamin B_{12} was then tested with rats on the heated diet. Twelve animals were used for this purpose. Their growth curves had been plateaued for about three weeks before the supplementation was started. As shown in Figure 2, sucrose and vitamin B_{12} did not give any response, but casein had a good growth-promoting effect, which became greater as the dosage was increased from 0.25 g to 1.0 g per day and rat.

The results of further investigations into the growth-restoring effect

of casein supplementation are shown in Figure 3. A comparison with the figure 1 shows that there is a good agreement between the growth rate of rats on the heated diet supplemented with 1 g (Fig. 3: 1000 mg) of casein per day and that of rats on the unheated diet.

Finally, the following of the component amino acids of casein were tested as supplements to the heated diet: histidine, methionine, leucine and lysine. Of these only lysine was found to have a growth-promoting effect and the steepness of the growth curve on the 100 mg/day dosage was found to be somewhat less than that of the casein-supplemented rats. The curves are shown in Figure 4.

Discussion.

These experiments show that the overall effect of mild heat treatment upon the dried and mixed components of a diet corresponding to the average composition of that used by the population in Finland is a definite loss of its growth-promoting value when tested with rats. The growth is, however, restored by the addition of casein or lysin. Evidently the destruction of lysin is to be considered as the main cause of the loss of the nutritive quality of the heated diet. This is in good agreement with earlier work on the detrimental effect of heat on dietary constituents (McCollum and Davies 1917, Greaves, Morgan and Loveen 1938).

Knowing that the destruction of the nutritive value of proteins and their components is dependent on several essentially different factors, such as the presence or absence of reducing sugars, moisture etc., the obvious destruction of lysin in the diet used in this experiment by the relatively short exposure to 140° C could not beforehand be taken for granted. The present observations none the less demonstrate that the essential nutrients may seriously suffer from heating, even under conditions where the composition of the diet is very near that which is customary in every-day life. Whether such a deleterious heating effect would result in insufficiently balanced diets under ordinary household conditions cannot be ascertained on the basis of the present data, the common processing of human food being in some respects different from the method used in this study. Such a dietary imbalance need naturally not cause any serious deficiency symptoms but might rather manifest itself, *e.g.*, in a decreased fitness and a lower resistance towards deleterious external influences.

Summary.

The effect of mild heat treatment on the nutritional value of a diet composed to correspond to the average composition of the diet of the Finnish people has been investigated. The test diet was dried and then kept at 140° C for 30—40 minutes. Rats were used as test animals.

The heated diet caused a total cessation of the growth of the rats. The growth was restored by the addition of casein or lysin. Leucine, methionine, histidine, vitamin B_{12} and sucrose were without effect.

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The Effect of Light on the Appearance and Development of the Electroretinogram in Newborn Kittens.

By

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Received 1 September 1955.

In order to study the effect of light on the appearance and development of the electroretinogram (ERG), newborn kittens have been examined in this work. Only the first part of the ERG, comprising a- and b-waves, has been studied. A group of kittens born and bred in normal daylight as well as a group born and bred during the first few weeks of life in darkness has been examined.

Investigations on the ERG in newborn babies have shown that during the first few days of life this is absent or else very small. The b potential of the ERG then increases slowly in size during the first 4—6 months of life (ZETTERSTRÖM 1951). In the same work the question of whether light favours the development of the ERG was discussed. During investigations on premature infants (ZETTERSTRÖM 1952), in which the appearance of an ERG is delayed, it was found that the b potential arises in advance of the expected time of birth, a fact which suggests an external stimulating factor, most likely to be illumination. In the present work this hypothesis has been put to an experimental test.

Methods.

The normal material consisted of a group of 36 kittens with a weight at birth of 100—150 gr., bred by their mothers. The ERG of these kittens have been observed from the day of birth until an age of about ten weeks.

The control group, with the same average weight at birth, comprised 32 kittens, born and bred in total darkness. By means of special channels the mothers were given an opportunity of spending a few hours daily in the light. They also had a daily dose of 400 I.E. vitamin D and 1000 I.E. vitamin A in their food. The first ERG measurements in these animals took place between the 6th and 49th day of life.

The kittens bred in darkness were conveyed to the investigation in light-proof boxes. Before the experiment all the animals were anaesthetised with nembutal, 40 mg/kg body weight, and had pantocaine dropped into the conjunctival sac. The pupils were dilated by a drop of 1 % homatropine.

Kittens open their eyelid fissure spontaneously on the 6th to 10th day of life (LANGWORTHY 1929). When experiments were carried out before the spontaneous separation had occured the bloodless membrane which holds the eyelids together was carefully cut apart, and, when for technical reasons necessary, the medial nictitating membrane was extirpated. The cornea, then revealed, was somewhat cloudy during the first days of life. This did not prevent a definite but slow contraction of the pupil when, as early as during the first day of life (ABELSDORFF 1905) it was exposed to strong light.

It is not possible, however, during the first two weeks of life, to examine the retinal fundus in detail. To compensate for the absorption of light in the cloudy cornea 800 lux was selected as stimulus light. This was found to give maximal readings. Series with 20, 80, 800 and 1200 lux respectively have been made but it transpired that in the majority of cases less than 800 lux was required to give a maximum reading. KARPE and VAINIO-MATTILA (1951) have shown that ERG recordings in human eyes with light-absorbing media cloudy in consequence of cataract may require stronger stimulating light than the normal 20 lux in order to give maximal readings.

The ERG was recorded according to the method of KARPE (1945). The indifferent electrode, was, however, for technical reasons placed in the ear of the cat. All the animals examined, with one exception, awoke 1—3 hours after the commencement of the anaesthesia.

Results.

With regard to kittens born in daylight there was no ERG at all during the first 5 days of life. Between the 6th and 10th day a small but nevertheless measurable ERG appeared. The b potential then increased with age and at an age of 3—4 weeks it reached 0.10—0.25 mV. The type of ERG also changed. The rate of rise of the b potential

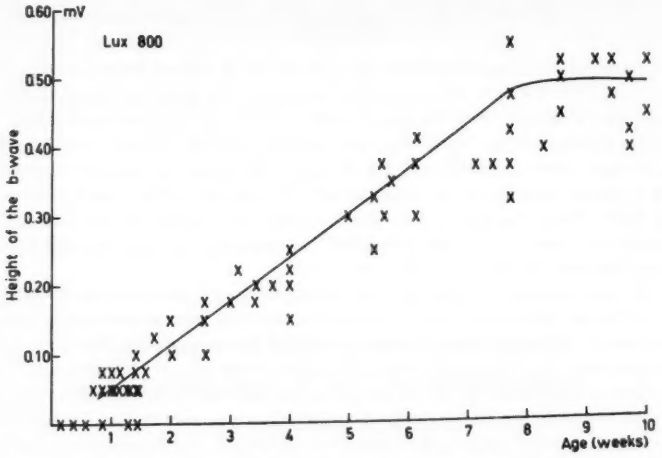

Fig. 1. The development of the *b* potential of the ERG in kittens bred in daylight during the first 10 weeks of life.

became faster and its duration shorter. No definite a-wave could be observed. A four weeks' old kitten which was too deeply anaesthesised and ultimately died, displayed, however, only a negative reading. This suggests that the negative component P III has developed at this stage but requires elimination of the positive components in the ERG, P I and P II, in order to become visible.

In 5—7 weeks' old kitten the b potential was 0.25—0.40 mV, and at an age of 9—10 weeks, the ERG in every respect agreed with that of grownup cats, being 0.40—0.60 mV (see Fig. 1).

In Fig. 2 the development of the b potential of the ERG in kittens born and bred in darkness is shown graphically. The time of appearance of the first ERG potential was considerably delayed in comparison with that of kittens bred in daylight. For kittens bred in daylight there was no recordable ERG in 17 % during the first 4 weeks of life, and for kittens bred in darkness there was no recordable ERG in 63 % during the same time. In those cases where a potential had arisen in spite of the animals living in darkness a prolongation of the latent period, *i.e.*, the time between the stimulation with light

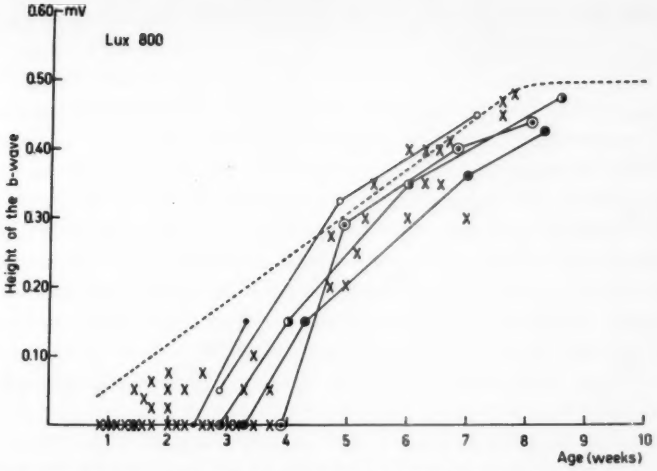

Fig. 2. The development of the b potential of the ERG in kittens bred in darkness during the first 10 weeks of life. The continuous lines indicate the same test animals at several investigations. A cross (×) is the result of the first investigation on different animals. The dotted line indicates the development in kittens bred in daylight.

and the commencement of the b-wave, was observed at recordings made during the 2nd and 3rd weeks of life, as appears from Table I:

Table I.

Latent period of the b-wave in seconds.

Age of kittens	12 days	17 days
In daylight.....	0.08 — 0.12 sec.	0.06 — 0.10 sec.
In darkness.....	0.14 — 0.17 sec.	0.12 — 0.14 sec.

The latent periods in the different animals are shown graphically in Fig. 3. If the eye had been but once exposed to a strong source of light, the development of the b potential started and it rapidly reached the same size as in a kitten of the same age bred in daylight.

It has, however, not been possible by means of a sojourn in darkness to delay for more than about 3 weeks the appearance of the ERG. In kittens which remained in darkness until they were more than

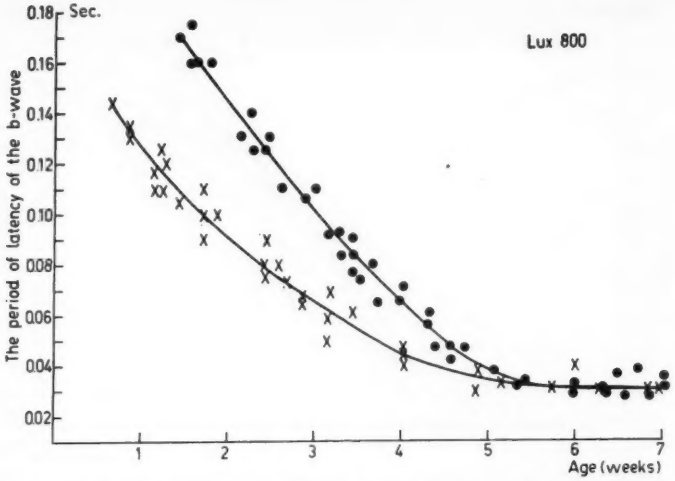

Fig. 3. The latent period of the b-wave during the first 7 weeks of life. Dots indicate animals bred in darkness and crosses animals bred in daylight.

4 weeks old before the first exposure to light took place, a definite ERG, normal for the age, could be recorded.

These differences between kittens in the dark and kittens in daylight vanished after one experiment with light. This implies that the eye had been exposed around 30 times in three hours to very brief flashes of 800 lux.

Discussion.

The retina of the newborn infant is, apart from the macular region, rather well developed histologically. The newborn kitten, on the other hand, has an undeveloped retina. This explains the circumstance that the ERG can be recorded at the earliest at the end of the first week of life. SCHULZE (1866) considered that in the retina of the newborn kitten rods and cones were non-existent. ABELSDORFF (1905) however, divided it into three zones, one completely rod-free situated along the periphery, one comprising the whole rear section of the eye, except for the centrum, where distinguishable rods of undeveloped type are noticeable, and one small central zone with clearly formed

cone and rod cells. This histological picture appears more probable in view of the fact that light-receptors obviously are present, since the pupil reflex can be released as early as during the first day of life. SIDMAN and WISLOCKI (1954) have shown that during the first day only the rods in the central part of the retina have formed segments, but as early as at an age of 6 days all rods display segments which, although still short, are clearly distinguishable and which successively become longer. This is a development which can be correlated with the appearance of the ERG after about the 6th to 10th day of life and with the subsequent increase of potential. The histological development takes place from the centre towards the periphery in the shape of a slowly increasing mature area with a larger and larger diameter, which finally comprises the whole surface of the retina.

Areal investigations on the retina of fully-grown cats (WIRTH and ZETTERSTRÖM 1954) have shown that in order to obtain a maximal ERG of normal type it is necessary that an area with a diameter of at least 3—5 mm should be exposed to light. A very small and slow response of the same appearance as in newborn infants and in kittens at the end of the first week was obtained when stimulating an area with a diameter of 1.5—2.0 mm. When stimulating areas with diameters of less than 1.5 mm, no ERG could be obtained. These facts support the notion that the appearance and development of the ERG is partly dependent on the size of the area, *i.e.*, that the number of active receptors during the first days of life are too few to give rise to an ERG.

This circumstance, could also explain the extremely slow pupil reflex in the beginning, which becomes rapid within the course of 14 days.

The appearance of the ERG has been shown to be delayed, 28 days at the most, in kittens bred in darkness. It is obvious, however, that the effect of light on the development only during a limited time can be a contributary factor. Kittens kept in darkness longer than 28 days have, in spite of this, shown an ERG normal for their age and with a normal latent period. The process of development of the retina after this age clearly progressed normally irrespective of the illumination. In 11 of 32 kittens bred in darkness no definite delay in the appearance of the ERG was demonstrable. The latent period to 800 lux of the very first ERG has however, always been delayed in comparison with that of kittens of the same age bred in daylight.

The fact that the latent period for the animals bred in darkness is delayed shows that the light, in one way or another, is of fundamental significance for (one or more of) the factors which accelerate the presumed chemical processes in the retina or its ERG. However, the present results do not give a definite indication of the nature of these factors.

The hypothesis put forward was that light might favour enzymatical processes in the retina, resulting in rods being able to convert light energy into chemical energy and thence into electrical energy. BIRUKOW and KNOLL (1952) have shown that they could delay the differentiation of the rod elements in small larvae of frogs by letting these live in darkness.

With the aid of histochemical technique SIDMAN and WISLOCKI (1954) have demonstrated that in the retina of a kitten, one day of age, the rods, which have just begun to grow out beyond the limiting membrane, stain very weakly for sulfhydryl groups. — In the retina of a kitten, six days of age the newly formed outer segments of the rods stain weakly. The choroidal tapetum, the external limiting membrane and the outer plexiform layer all stain more deeply than the rod outer segments, in contrast to the observations on adult retinas, where outer segments show the most intense staining.

Through investigations made by LÜNEN (1952/53), by JONES, BLACK, FLYNN, and LIPMAN (1953) and others it has been shown that the presence of SH-groups is necessary for intra-cellular energy transport.

ARNON, WHATLEY and ALLEN (1954) have shown that the light energy in chloroplasts is converted to phosphate-bound energy, adenosine triphosphate. Such a phosphorylation might in animal tissues as well as in green plants require the intermediation of SH-groups. In view of the similarities which presumably exist with regard to the conversion of energy taking place in the retina and in green plants it is probable that also in the retina light energy is converted to phosphate-bound energy via SH-groups.

As it is thus probable that phosphorylation is required in order before an ERG can arise and, furthermore, the development is delayed when the animals are kept in darkness, it would be interesting to investigate at what point SH-compounds begin to occur in animals bred in daylight and animals bred in darkness. Would there be a correlation with the facts presented above?

Summary.

In order to determine the effect of light on the development of the ERG, this response has been recorded in kittens bred in darkness and in kittens bred in daylight.

The appearance of an ERG in kittens bred in darkness in the majority of cases was found to be considerably delayed. The latent period of the b-wave of the ERG in animals bred in darkness was likewise prolonged in comparison with those bred in light.

The significance of these results has been discussed.

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Comments on H. Dahlström's Criticism of Sjöstrand's Method of Indirect Determination of the Carboxyhemoglobin concentration in Man.

By

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In this journal DAHSTRÖM (1955) has published a critical survey of the present author's method for determining the COHb-concentration in the blood by analysis of the CO- and O_2-pressures of the alveolar air, and also his method, based on the same principle, for determining the total amount of hemoglobin and blood volume. (SJÖSTRAND 1948 a and b). In 1953, DAHLSTRÖM first presented the results of his investigation at a meeting of the Physiologic Society in Stockholm. Later CARLSTEN and coworkers (1954) checked earlier reports (SIÖSTEEN and SJÖSTRAND, 1951) on the question whether the proportionality constant M in Haldane's formula could actually be used as a constant for determination under given conditions. By means of determinations on 55 subjects of the CO-concentration of alveolar air, before and after the administration of small volumes of carbon monoxide, and of the COHb-concentration in blood samples taken at the same time and analysed by three different methods, the variations of M between different subjects were found to show complete conformity with the variations that might be expected from the errors inherent in the method for determining M. Furthermore, M disclosed the same variations in duplicate determinations on one and the same subject as on different subjects.

DAHLSTRÖM has presented in the mentioned paper a selection of his results as described earlier in the lecture. He does not enter into an objective analysis of the experiments performed in the meantime by CARLSTEN and coworkers, giving an obviously erroneous account of their results. DAHLSTRÖM's statements are misleading also concerning other previous investigations. However, the present author will confine himself to a critical analysis only of DAHLSTRÖM's investigation in order to try to ascertain why the results now published by DAHLSTRÖM differ from earlier ones.

The Error in the Determination of M.

A determination of M and its variations between individuals, if any, at low COHb-concentrations *in vivo* is principially complicated by the comparatively big errors inherent in the methods that can be applied at the particular concentrations required in the present case. However, by determining the errors in all the methods used for a determination of M on a sufficiently large number of subjects, it is possible to ascertain whether the variations of M correspond to the errors of the method, or whether the value of M shows other variations.

In his work, DAHLSTRÖM used a modification of the method adopted by SIÖSTEEN and SJÖSTRAND (1951) to determine the COHb-concentration in blood samples. According to this modification, larger blood samples were employed, CO being released by H_2SO_4 instead of by potassium ferricyanide and the extraction of CO taking a longer time than by the original method. The error of this method is established by DAHLSTRÖM after determining the released CO-volume in five blood samples saturated with CO, at least two determinations being duplicate for one and the same blood sample. In the discussion, DAHLSTRÖM wrongly contends that the error of the method is based on 7 determinations. A comparison between extracted and calculated CO-volumes revealed the following deviations, expressed in per cent of CO-volume: $+2.5$, -2.6, $+2.6$, $+0.8$ and -0.3. From this DAHLSTRÖM concludes that the error of the method for a determination of COHb in calculating M equals between 2 and 3 per cent. Several arguments may be advanced against such a conclusion:

1) Neither the maximal error variations nor the standard error of a method can justifiably be ascertained from 5 determinations.

2) No proper conclusions can be drawn from a determination on blood samples saturated with CO regarding the error of the method in unsaturated samples. In calculating M in the material described in DAHLSTRÖM's paper, a determination on blood samples was made with, as a rule, 1—5 % of the saturation value. In order to obtain the same CO-volumes, the size of the blood sample must, in some test subjects, amount to as much as 100 times that used in determining the error of the method, *i.e.* 200 ml. However, DAHLSTRÖM did not mention the size of the blood samples used in the various determinations. Still, he gave an account of 2 determinations in samples of 20 ml with a COHb-concentration of approx. 10 % of the saturation value, finding considerably greater deviations between observed and calculated CO-volumes, *i.e.* — 2.9 and + 4.8 %, respectivly. He explained these greater deviations, as follows: "The amount of blood used was measured less accurately with a syringe, which may have introduced some error in these experiments". In order to be able to refer the largest deviation from the maximal deviations found in saturated blood samples to a lack of accuracy in measuring the blood sample, the error in measurement must be supposed to have equalled over 0.4 ml. However, another explanation of the greater deviations between observed and calculated values in the unsaturated blood samples may be derived from the fact that the error is greater at a lower COHb-concentration than at a higher one. This was, moreover, pointed out by SIÖSTEEN and SJÖSTRAND (1951). They reported a standard error of 2.6 % at a COHb-concentration of 10—100 %, of 4 % at a concentration of 2—10 % and of 6.4 % at a concentration of 0—2 %. The same is, broadly speaking, to be noted also when the released CO-volume is kept constant. This may be attributed, *inter alia*, to the fact that the hopcalite in the CO-meter is affected by other gaseous substances than CO, and that CO can be produced by oxidation of hemoglobin to methemoglobin (SJÖSTRAND 1951). This CO production may be expected to vary under different circumstances.

3) A fair conformity between the calculated and the observed CO-volume in CO-saturated blood samples does not justify the conclusion that CO is not produced at oxidation of hemoglobin to methemoglobin in only partly saturated blood samples. For, a production of CO at an oxidation of the hemoglobin seems to presuppose the presence of free oxygen. Any produced CO is, moreover, included

in the blank values added by DAHLSTRÖM to the administered CO-volume in the two tests with not CO-saturated blood samples. Such a CO production may explain why he, particularly in the blank determinations, obtained a remarkably higher COHb-value at the direct determination of COHb in the blood than at the indirect determination by means of the alveolar CO- and O_2-partial pressures. Also in the determination made by CARLSTEN and coworkers, a similar difference was observed in the use of H_2SO_4. The more hemoglobin contained in the analysed sample, the greater the volume of CO that may be produced. Accordingly, this error may be expected to show variations in DAHLSTRÖM's determinations.

4) It is not justified to conclude from an assumed error of the method of 2—3 % in a determination of the CO-volume extracted from the blood sample, that the COHb determination is done with the same accuracy. The COHb determination includes an error also in the determination of the CO capacity of the blood. DAHLSTRÖM ascertained this CO capacity from the hemoglobin concentration, without noting the error in the determination, nor the error involved in transforming the colorimetrically determined hemoglobin concentrations to CO capacity values. The total error in this determination may be assumed not to fall below a standard deviation of $\pm$ 3 % in a determination of the blood from different subjects. This implies that the error of the method in the COHb-determination should amount to over 4 %, even taking into account DAHLSTRÖM's assumed error of the method for determining the extracted CO-volume.

For a calculation of the experimental error in the recorded M-values, apart from a knowledge of the standard errors of the COHb-values, also the errors involved in determining the alveolar CO- and O_2-partial pressures have to be ascertained. Still, DAHLSTRÖM does not give any data concerning these errors. In a determination of pO_2 with Beckman's O_2 analysing apparatus, the standard error of the method may be assumed to equal about $\pm$ 3 %. The accuracy of the determination of pCO in the alveolar air is dependent on a series of factors, viz, the tightness of the rebreathing device and the time for the rebreathing which will be discussed later. Under favourable conditions, the error of the method generally equals 3—4 % at the particular concentrations concerned in this case. When, in a calculation of M, the total error of the recorded values is determined, it is found to equal about $\pm$ 6 %.

As regards the M-values published by DAHLSTRÖM, 9 out of 20 do not correspond to the mean ± 3 times this standard error. However, the fact that the error of the method is greater than that calculated in the above-mentioned way seems evident from determinations on two subjects in whom M varied considerably before and after an administration of CO, corresponding to an increase of the COHb-concentration by barely 2 %. In both these instances DAHLSTRÖM admits that the error in determining the COHb-values was greater than in other cases. He therefore leaves them out of the discussion, stating that »the blank values were unexpectedly low». However, these values equal 0.96 and 1.16 %, respectively, and are rather to be regarded as high in non-smokers, as also all the other values, before CO-administration, referred to by DAHLSTRÖM. They also differ but slightly from other values that have been accepted by him. If the blank values had been higher, the differences between the M-values would, in addition, have become still more marked. With the same justification as the forementioned values, also others can be excluded, *e.g.*, in the two test subjects who revealed unexpectedly high L-values. *As the errors of the method were not determined by* DAHLSTRÖM *in a mathematically and technically acceptable way, he was forced to arbitrary exclusions and attempts to explain away values that constitute irrefutable proof of the inaccuracy of the assumed errors of the method.*

DAHLSTRÖM states regarding the L-values that the error equals ± 5 %, without specifying the manner in which this was calculated. Even when the very low errors for the various methods are reckoned with as stated above, the standard error for L amounts to ± 8 %.

The Rebreathing time.

For a determination of the total amount of hemoglobin, in accordance with the present author's mode of procedure, as well as for a determination of M under similar conditions, an equilibrium is required between the pCO in the blood, the alveolar air and the sampling bag of the rebreathing apparatus. Such an equilibrium is obtained by adapting the rebreathing time so as to make the CO concentration in the system constant. The rebreathing time must be determined for each rebreathing device. It also has to be adapted to the material under examination, seeing that it has been found to be longer, for instance, in a group of patients than in children and athletes. DAHL-

STRÖM *does not seem to have paid any attention to this absolute requirement in determinations of M* as well as of the total hemoglobin content, to judge from his acceptance of a lack of equilibrium as an explanation of the varying M- and L-values.

The rebreathing time for attaining an equilibrium is determined, *inter alia,* by the volume of the respiratory device, its construction and by the particular site in the system for administration of carbon monoxide. As regards the construction, DAHLSTRÖM states that the apparatus used in Stockholm was provided with a powerful blower instead of with respiratory valves. It has been observed in such cases that the rebreathing time has then to be prolonged, probably owing to the rapid mixing in the system of the administrated CO-volume. *The rebreathing times used by* DAHLSTRÖM *varied between* 15 *and* 21 *minutes after a supply of carbon monoxide. This time is generally too short,* even when particular care is taken to perform a suitable administration of carbon monoxide in the opinion of the present author and other investigators (cp. for instance HALLBERG 1955). At the present author's laboratory, a rebreathing time of 30 minutes is used and it has been observed in individual cases, such as in certain lung diseases, that even this period may be too short.

It emerges from the results obtained by DAHLSTRÖM that he failed to establish such an equilibrium. This may explain for instance, why the M-values after an administration of carbon monoxide are lower than before, and the L-values still lower. No significant difference was noted between these values in the investigation carried out by CARLSTEN and coworkers on a much more comprehensive material. Another contributory cause of the marked difference between M- and L-values in DAHLSTRÖM's material may, moreover, be the above-mentioned possibility of a production of carbon monoxide when determining the COHb-values.

The Tightness of the Rebreathing System.

In order to reach an equilibrium between the CO-concentrations of the rebreathing system, the alveolar air and the blood, it is necessary that the employed rebreathing device and its connection to the test subject are tightly fitting. Intermittent leakages retard and prevent equilibrium. An insufflation of air through the leakage can be detected by a determination of the O_2-concentration in the system. To judge

from the alveolar pO_2-values denoted by DAHLSTRÖM, such an insufflation occurred in several cases. Cases of that kind are excluded in a determination of the total amount of hemoglobin. Also a leakage from within the system and outwards seems to have taken place in DAHLSTRÖM's experiments. Accordingly, when the blood volume is calculated by means of the values given by DAHLSTRÖM for the administered CO-volume (20—30 ml), as well as the COHb-values determined in the blood and the hemoglobin concentration of the blood, an average of 7.65 litres is obtained for the 10 test subjects, which corresponds to the blood volume of a man of normal growth with a body weight of about 100 kg. When the administrated CO-volume is equalled to 25 ml, one of the test subjects obtains a blood volume of 12.5 litres, another 10.5 litres and a third 9.9 litres. When determinations are made on the COHb-values calculated by the pCO in the alveolar air, *i.e.* in the way adopted for determination of the total hemoglobin, still higher values are obtained and an average value of 8.83 litres, the largest value being 15.7 l. These blood volumes are excessive and indicate that carbon monoxide has either leaked out in the course of the determination, or that DAHLSTRÖM has not performed the determinations according to his report, viz. "similar to the procedure in SJÖSTRAND's method for determination of total hemoglobin". *In all the experiments, only two otherwise satisfactory ones yielded a result regarding the blood volume that corresponds to the values generally obtained in calculating the pCO of the alveolar air, i.e. on Cr and Ka. These values show fair conformity with each other and with the M- and L-values arrived at by* CARLSTEN *and coworkers.*

Selection of Experimental data.

Considering that DAHLSTRÖM's method for determination of the COHb-concentration in the blood has, in all likelihood, a much greater error than stated by him, and that he failed to check whether an equilibrium had been obtained between the concentration of carbon monoxide in the rebreathing system, the air of the lungs and the blood in taking the blood and gas samples for analysis, and, further, that he did not check that the rebreathing device was tight in itself or at the connection to the test subject, it is not surprising to find marked variations between respectively the M- and L-values in different individuals. It is more remarkable, in fact, that they did not

vary still more. However, DAHLSTRÖM has pointed out earlier in the above mentioned lecture that he observed both lower and higher M-values than those reported by him in the publication, but he does not mention the principle according to which he made his selection of values for the publication. In a study of the distribution of the M-values, this selection was found to be hardly at random. Thus, the M-values are distributed in 2 groups, as though the mean of M were calculated for each individual in both determinations, lying within the respective limits values of 200—250 and 300—400. Between 250 and 300 there is only one value, *i.e.* one of the values rejected by DAHLSTRÖM. It seems as though the M-values are distributed in a moderately low group and in a moderately high group. In the normal cases examined by DAHLSTRÖM but not defined by him more than by the limit values of M, the variations of M seem to have been considerably smaller, lying entirely within 3 times the mean error calculated above. On the other hand, *not less than* 10 *of the* 20 *more closely described determinations exceed the upper limit value in this normal material, although they too were performed on normal cases or subjects prepared for minor operations.*

Without access to all of DAHLSTRÖM's investigation data, it is impossible to decide whether he was more objective in selecting cases for publication than in presenting other data and referring to investigations made by others.

Summary.

DAHLSTRÖM's criticism of the present author's method for determination of the COHb-concentration of the blood *in vivo*, by calculating the pO_2 and pCO of the alveolar air, and the method based on these values for determination of the total amount of hemoglobin and blood volume does not hold good for the following reasons:

DAHLSTRÖM has not determined satisfactorily the error in the methods employed by him for calculating the proportionality constant of the relation between the alveolar pCO and the COHb-concentration of the blood.

DAHLSTRÖM has not, apparently, paid any attention to the establishment of an equilibrium between the concentration of carbon monoxide in the rebreathing system and the blood.

To judge from DAHLSTRÖM's statements, a leakage must have

occurred in the rebreathing system or at its connection to the test subjects.

The values published by DAHLSTRÖM represent a selection from a bigger material, without his specifying the principles of that selection.

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Efficiency of Pulmonary Ventilation during Rest and Light Exercise.

A Study of Alveolar Nitrogen Wash-out Curves in Normal Subjects.

By

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Received 2 September 1955.

Since KEITH (1900) and TENDELOO (1902) proposed the theory of unequal ventilation of the lungs half a century ago, this problem has been subject to a large number of investigations. The earlier literature has been critically reviewed by RAUWERDA (1946). Uniformity of alveolar ventilation can be defined as the condition in which the inspired air, of homogeneous composition, is distributed proportionally equally to all lungspaces and mixes ideally with the pre-inspiratory gas in the alveoli. Even in many normal persons, however, this situation does not always exist (ROELSEN 1939, DARLING, COURNAND and RICHARDS 1944, ROBERTSON, SIRI and JONES 1950). That imperfect mixing within a single alveolus plays any important part in explaining the non-uniformity of alveolar ventilation is improbable (RAUWERDA). Two other mechanisms appear to be more important. The first of these is unequal expansion of different parts of the lungs, i.e. differences in percentage volume increase during inspiration. The second possibility is FOWLER's (1952) "sequential ventilation": due to volume changes asynchronous to the active respiratory movements there is a preferential distribution of dead space gas early in inspiration to some parts of the lungs.

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Several recent studies, reviewed by FOWLER (1952), have been concerned with the extent of unequal alveolar ventilation under normal and pathological conditions. There are conflicting opinions about the influence of an increase in tidal air, through voluntary hyperventilation or exercise, on the intrapulmonary gas distribution:

1) Ventilation becomes more uniform: RAUWERDA (1946), FOWLER (1949), TIEMANN (1933), MUNDT et al. (1941).
2) Efficiency sometimes improved, sometimes lowered; in general less influence on ventilatory efficiency than expected: ROBERTSON et al. (1950).
3) Ventilation becomes less uniform: ROHRER (1915), SONNE (1936—1937).

ROBERTSON et al. (1950) find that voluntary hyperventilation and the hyperventilation caused by exercise are not as efficient as would be predicted from the increase in ventilatory minute volume, because some portions of the lungs remain poorly ventilated. With moderate increases in minute volume the efficiency is even lowered, but with high minute volumes it is increased.

The term "ventilatory efficiency", as used throughout this paper, refers to the efficiency of nitrogen wash-out from the lungs during oxygen breathing and thus to the efficiency of the turn-over of gas molecules in the alveoli. It may be expressed in several ways. In this study, two independent indices of ventilatory efficiency have been used, as will be discussed below (see Treatment of the experimental data).

We decided to study the problem of the ventilatory efficiency of hyperventilation by measuring the nitrogen percentage in the end-tidal air (i.e. the last portion of expired air) during oxygen breathing at rest and during light and moderate exercise. The justification for using end-tidal, and not mean expired concentrations of nitrogen is to be found in a series of experiments made by LUNDIN (1955). He found that in normal subjects the nitrogen percentage of the end-tidal air during oxygen breathing was proportional to the amount of nitrogen washed out per breath (= mean expired concentrations times tidal volume). The plots of endtidal concentrations and of amount of nitrogen given off per breath, determined simultaneously with two nitrogen meters, both on a semilogarithmic scale, ran parallel to one another. From this it was concluded that in normal subjects the end-tidal percentages could be used as an accurate

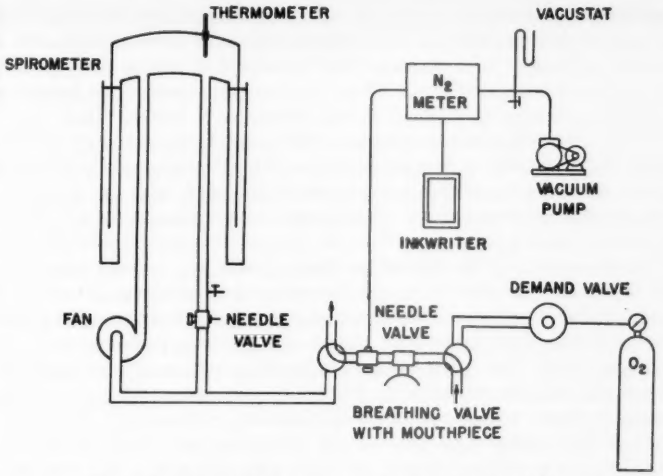


Fig. 1. Experimental arrangement. See text for description.

method to follow the quantitative course of the nitrogen wash-out from the lungs, in spite of theoretical objections (FOWLER, personal communication). Stated briefly, these objections are that changes in expired alveolar gas concentration in the course of one expiration are neglected, and that an eventual preferential distribution of dead space gas is not taken into account. Judged by LUNDIN's experiments, these factors, however, appear to be of minor importance in the normal subjects studied.

Methods.

The experimental arrangement is shown in Fig. 1. Before each experiment the subject relaxes in a semi-reclining position to obtain a relatively basal state. He is then connected to the mouthpiece and breathes room air through a respiratory valve (VON DÖBELN 1949) without dead space. The expired air passes a needle valve through which a small gas sample (20 ml./min.) is continuously sucked to the gas analysis apparatus, a nitrogen meter (LILLY, ANDERSON and HERVEY 1943), modified by LUNDIN and ÅKESSON (1954). This apparatus permits an almost instantaneous analysis of the expired air; the output of the meter is recorded with a fast Esterline-Angus ink writer. The total response time is 0.2 sec. The accuracy of measurement is $\pm$ 1 % between 80 and 15 % nitrogen, and $\pm$ 0.1 % between 15 % and zero. When

breathing is observed to be quiet, the subject is changed over from room-air to oxygen, delivered from a tank through a demand valve, at the end of a normal expiration. With the same valve (for technical reasons the two parts are shown disconnected in the figure) the expiratory side of the breathing circuit is connected to a closed circuit, containing a 100-liter Tissot spirometer, a circulating blower (capacity 150 l./min.), and a second needle-valve. After the start of oxygen breathing (99.7 % oxygen and 0.3 % inert gases) the nitrogen percentage in expired air falls and these concentrations are recorded on the ink writer. The volume of expired gas in the spirometer is recorded on a kymograph. When the nitrogen concentration in end-tidal air is down to 2 % the subject is changed back to room air breathing. The time from the start of oxygen breathing up to this point is measured and called wash-out time. After the wash-out period the nitrogen percentage in the accumulated expired air in the spirometer is measured with the nitrogen meter. The meter is checked by HALDANE analysis in duplicate for each series of experiments, which for each subject consists of one resting and two work experiments in succession, with an interval of about 15 minutes between each two of them. The work performed consisted of leg-lifting to a constant height. In each series one experiment was done with a frequency of the leglifting of 56 per minute, and another with a frequency of 112 per minute. These frequencies were checked by a metronome.

We did not try to measure the amount of work or the oxygen consumption. It is probable that there is a rather considerable variation between different subjects in the amount of work necessary for this kind of exercise. The heavier work gives an average increase in respiratory minute volume of about 100 % and an increase in tidal volume of the same magnitude. The breathing frequency did not change appreciably, on the average. The subjects were 9 students and laboratory employees in good health, their ages ranging from 17 to 44 years.

Treatment of the experimental data.

The formulas used are the same as those of FOWLER *et al.* (1952). The basic equation (DARLING *et al.* 1944) describing the fall in nitrogen concentration during oxygen breathing in an evenly ventilated system is:

$$F_{A_n} = F_{A_o} w^n \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where F = fractional nitrogen concentration.

F_{A_o} = alveolar nitrogen concentration before oxygen breathing.

F_{A_n} = alveolar nitrogen concentration after n breaths of oxygen.

$w = \dfrac{V_L}{V_L + V_T - V_D}$ is called the alveolar dilution factor.

V_L = volume of the lung at the end of an expiration = functional residual volume.
V_T = tidal volume.
V_D = respiratory dead space.

This equation, however, holds good only for lungs with an ideal ventilation, *i.e.* behaving as a single bellows system. In such a system the nitrogen concentration falls exponentially during oxygen breathing: with every inspiration the concentration is multiplied by the factor w, which is smaller than 1.

In many normal subjects the experimental data do not conform to this formula. The calculations given by FOWLER *et al.* (1952) show that in these subjects the over-all ventilation behaves as the sum of two or three lung spaces, each ventilated at a different rate. When these are considered to be ventilated independently, in parallel, in accordance with the concept of regional inequality of lung ventilation, it is possible to define the volume and ventilation rate of each fraction. For this analysis a plot of mean expired nitrogen concentration is made on a logarithmic scale against number of breaths on a linear scale. As mentioned before, we did not, however, determine mean expired concentrations in our experiments, but end-tidal concentrations and we have used these end-tidal nitrogen percentages for the calculation of the ventilation fractions.

For normal subjects with more than one ventilation rate, equation (1) may be written:

$$F_{A_n} = F_{A_{1_o}} \cdot w_1^n + F_{A_{2_o}} \cdot w_2^n \quad \ldots \ldots \ldots \ldots \ldots \ldots \quad (2)$$

where w_1 and w_2 are alveolar dilution factors for two different fractions of the lung volume, and $F_{A_{1_o}}$ and $F_{A_{2_o}}$ are the fractional nitrogen concentrations in the end-tidal air belonging to these volumes at zero time.

w, however, is a fractional value, which means that for an increasing value of n the expression $F_{A_n} = F_{A_o} \cdot w^n$ goes toward zero, and this more rapidly for smaller values of w. This fact is used to separate the slower ventilating fractions from the more rapid ones. After plotting the F values on a logarithmic scale against n on a linear scale, a curve through all F dots represents F_{A_n} in equation (2); Fig. 2. The components are graphically determined by extrapolating the straight part of the curve towards zero and subtracting this curve from the total one. If one deals with two separate elimination rates of the lungs one obtains two straight lines representing the expressions:

$$F_{A_{1_n}} = F_{A_{1_o}} \cdot w_1^n \text{ and } F_{A_{2_n}} = F_{A_{2_o}} \cdot w_2^n.$$

The steeper line is the fraction with the smallest w value. It is assumed here that the inspired gas contains no nitrogen and that there is no transfer of nitrogen from blood and tissues. In our treatment of the measured values for F_A we have corrected for the 0.3 % nitrogen in inspired oxygen and for nitrogen given off from blood and tissues. We used the same

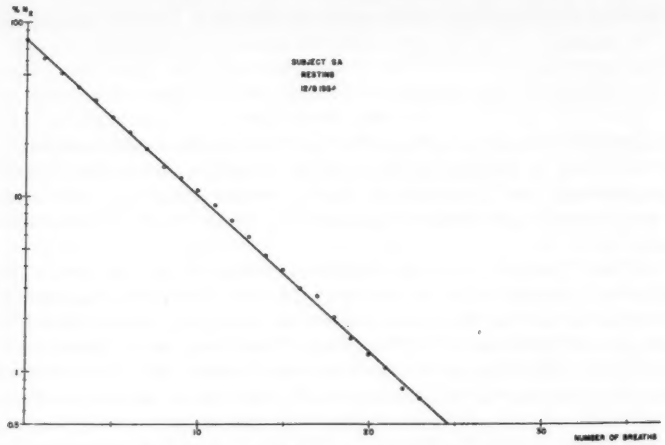


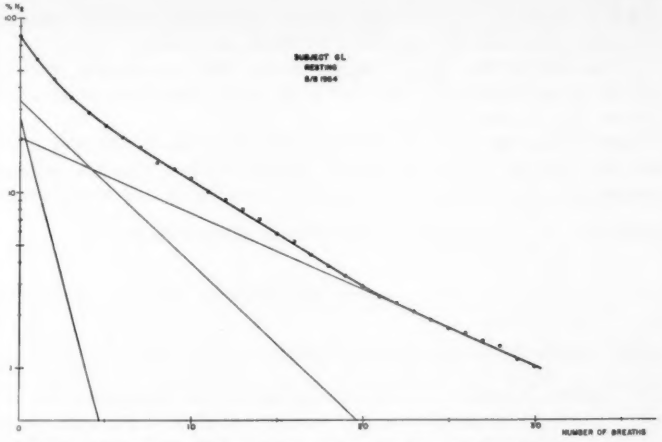
Fig. 2. Alveolar nitrogen clearance curves.
a) one ventilation rate present (uniform ventilation)

correction for tissue nitrogen for both rest and work experiments, *i.e.* 60 ml for the first, 45 ml for the second and 30 ml for the third minute, and the nitrogen elimination is assumed to start at the first breath and then decline to the average minute value in the middle of each minute. It is probable that the nitrogen elimination first increases during the first few breaths and then decrease but from a practical point of view, the nitrogen from the tissues being a small part of the total nitrogen eliminated, particularly during the first few breaths, we think it correct to assume an even fall in the amount of added tissue nitrogen. The actual values for tissue nitrogen mentioned above were taken from the experiments of LUNDIN (1953). For percentage correction we assumed a ventilatory dead space of 150 ml both for rest and work experiments.

From the analysis of the elimination curve and of the spirometer content the following data can be obtained: w_1, w_2; F_{A_1}, F_{A_2}; V_T, V_L. Then from these values f_1, f_2 .... can be calculated, *i.e.* the decimal fractions of the lung volume with the dilution factors w_1, w_2 ....

For these calculations, the amount of expired gas in the spirometer is measured and corrected to body temperature, ambient pressure, saturated (BTPS). From this volume and the total number of breaths V_T is obtained.

V_L, the functional residual air, is calculated by dividing the total amount of expired nitrogen, corrected for tissue nitrogen and nitrogen in inspired oxygen, by the fall in end-tidal fractional concentration of nitrogen, *i.e.* $0.8-0.02$; the volume obtained is corrected for instrumental dead space, To calculate $w_{1,\ 2,}$ ..... one reads the intercept of each straight curve.



b) three different ventilation rates present. Further explanation in the text.

drawn on the semilogarithmic plot of F_A against number of breaths, with the ordinate. This figure is F_{A_0} for that particular fraction. From the same curve, another value for F_A is read, *e.g.* at $n = 10$. With these data $w_{1,2} \ldots .$ can be obtained from:

$$F_{A_{1_n}} = F_{A_{1_o}} \cdot w_1^n,\ F_{A_{2_n}} = F_{A_{2_o}} \cdot w_2^n, \text{ etc.}$$

The volume for each fraction is found from $V_{L_{1,2} \ldots .} = \dfrac{F_{A_{1_o, 2_o} \ldots .}}{1 - w}$

This formula is derived from the mathematical expression for the sum of the terms of a decreasing geometrical progression. In its original form, this formula contains in the numerator not F_{A_n}, but the amount of nitrogen expired in the first breath for every fraction. As explained above we feel justified in replacing these values by alveolar fractional concentrations in normal subjects. Solution of this formula for $V_{L_{1,2} \ldots .}$ gives an arbitrary figure; from the sum of these for all fractions the percentage volume of the fractions, $f_{1,2, \ldots .}$ can be calculated. As the total lung volume is known, the absolute volume of the fractions, too, can be obtained. To permit comparison of the ventilation at rest and during work, and between different individuals, the following expressions for the efficiency of ventilation have been calculated:

1. Wash-out volume per liter F.R.A., *i.e.* total expired volume during the test, divided by F.R.A., both in liters, BTPS corrected. This quotient indicates the number of liters ventilation required to wash out each liter

of F.R.A. down to 2% end-tidal nitrogen concentration. This value has been called the lung-clearance index by BECKLAKE (1952).

2. The average number of breaths during which a nitrogen molecule remains in the lung spaces (FOWLER *et al.* 1952, ROBERTSON *et al.* 1950), and the ideal number of breaths.

These values need some explanation. FOWLER *et al.* (1952) have shown that the average number of breaths during which a nitrogen molecule remains in a system from which nitrogen is completely and evenly washed out equals $\frac{1}{1-w}$, in which w is the alveolar dilution factor; $w = \frac{V_L}{V_L + V_T - V_D}$. In a system with different washing out rates or uneven ventilation the number of breaths is $\frac{f_1}{1-w_1} + \frac{f_2}{1-w_2} \ldots$. This number of breaths is called the actual average breath number. It is also possible to calculate what this number would be if the same system (V_L and effecte tidival volume being equal) were uniformly ventilated. This is the ideal average number of breaths a nitrogen molecule remains in the lung space. It can be shown that $\frac{f_1}{w_1} + \frac{f_2}{w_2} + \ldots = \frac{1}{W}$, here W is the value of the alveolar dilution factor of the same system if it had been evenly ventilated; the ideal average breath number is then $\frac{1}{1-W}$. By comparing the actual and the ideal average number of breaths it is possible to assess the extent of uneven ventilation. In the ideal case, there is no delay: the actual number equal the ideal number of breaths. In most normal subjects, the actual number is larger than the ideal value; this difference is expressed in the delay percentage. The pulmonary nitrogen clearance delay in % is $\left(\frac{\text{actual average breath no.}}{\text{ideal average breath no.}} - 1\right) \times 100$.

Results.

Table 1 presents the results in 9 healthy male subjects. In the majority of cases the elimination curves can be divided into 2 phases. The mean fractional volume of the slow phase at rest is 0.82, with a mean w value of 0.865. The corresponding mean of the actual average number of breaths is 7.99, and the ideal number is 4.52 on the average in this series; the delay is 26%. For the two degrees of exercise the average delay is 31% for the light degree, and 20% for the heavier degree. These values show a rather large variation about the mean values, ranging from 0 to 66% for the rest experiments (Table I) and from 0 to 54% in the work experiments. This is to be expected from

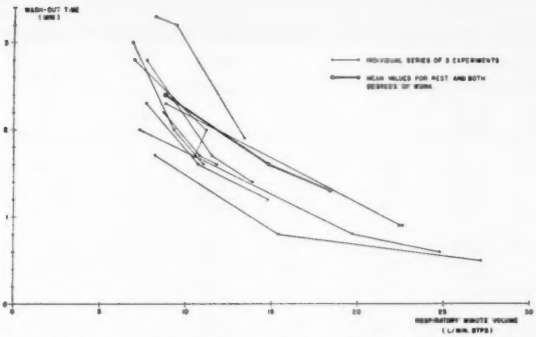


Fig. 3. Relationship between wash-out time and respiratory minute volume. Each thin line (———) refers to one series of 3 experiments (Table I); the heavy line (———) represents the mean values of Table I.

the method of calculating the clearance delay: as w for the greater part of the lung volume ranges grossly from 0.8 to 0.9, small differences in w will affect the expression $\frac{f}{1-w}$ to a larger extent. From the values obtained the efficiency of ventilation appears to be about the same at rest with a tidal volume of about 700 ml as during exercise with tidal volumes of around 1 000 and 1 500 ml, respectively.

One of the main advantages of this method of study, however, is the fact that more than one index of the extent of unequal ventilation can be obtained.

The values for the other index: the wash-out volume per liter functional residual volume, support the conclusion drawn from the clearance delay values. The mean values of this index at rest and during both degrees of exercise are 7.6 ± 1.0, 7.7 ± 0.96 and 7.6 ± 0.93 liters per liter F.R.A., respectively.

The increase in respiratory minute volume is mostly caused by an increase in depth of respiration; the washing-out time decreases as the minute volume increases, as is shown in Fig. 3.

To test the reproducibility the experiments were repeated 9 times with the same subject G.L. Table II shows the results. The clearance delay percentages are in the same range as found in the whole series:

Table

Subject	Age years	Work freq. /min.	Breaths /min.	Tidal volume ml.	Wash-out time min.	F.R.A. L.	Vol. 0_2/ L. FRA.
L. Å.	33	rest	14.0	620	2.2	2.5	7.6
		56	12.7	850	1.7	2.4	7.7
		112	14.4	820	1.6	2.6	7.3
A. B.	28	rest	15.3	452	3.0	2.4	8.8
		56	14.0	666	2.0	2.6	7.2
		112	13.8	794	1.6	2.1	8.3
S. A.	17	rest	8.8	931	1.7	1.9	7.5
		56	13.8	1118	0.8	1.8	7.7
		112	22.0	1237	0.5	1.8	7.8
O. N.	22	rest	13.9	502	2.8	2.7	7.5
		56	26.0	432	2.0	2.3	9.9
		112	8.2	1282	1.7	2.4	7.4
G. L.	44	rest	11.3	681	2.3	2.1	8.5
		56	13.1	818	1.6	2.1	8.2
		112	15.0	988	1.2	1.9	9.2
K-E. H....	22	rest	9.6	817	2.8	2.7	8.3
		56	7.7	1498	1.7	2.7	7.2
		112	7.2	1924	1.4	2.5	7.7
N. G.	22	rest	21.0	347	2.0	2.5	5.8
		56	25.0	788	0.8	2.0	8.1
		112	10.0	2475	0.6	2.1	7.1
R. J.	25	rest	15.1	543	3.3	3.3	8.3
		56	11.8	797	3.2	3.0	6.8
		112	12.1	1105	1.9	3.4	7.3
K. B.	23	rest	7.0	1263	2.3	3.1	6.5
		56	12.2	1856	0.9	3.0	6.8
		112	8.2	2735	0.9	2.6	7.8
Mean values.		rest	12.9	684	2.4	2.6	7.6
		56	15.1	980	1.6	2.4	7.7
		112	12.3	1499	1.3	2.4	7.6

Tables I and II: All experiments have been grouped in series of 3, one rest the frequency of leg-lifting per minute. The other values have been described in and saturation.

at rest 30 %, light exercise 29 %, moderate exercise 34 %. No significant difference is found between rest and exercise values, but the variability is again rather large. The amount of oxygen used per liter

I.

w_1	f_1	w_2	f_2	Average nr. of breaths N_2 molecules remain		
				actual	ideal	delay in %
0.904	0.72	0.635	0.28	8.10	5.22	55
0.856	0.61	0.612	0.39	5.23	3.85	36
0.875	0.55	0.689	0.45	5.85	4.55	29
0.992	0.72	0.753	0.28	10.36	7.58	37
0.860	0.84	0.667	0.16	6.48	5.62	15
0.830	0.89	0.399	0.11	5.42	3.89	39
0.720	1.00			3.57	3.57	0
0.671	0.80	0.479	0.20	2.81	2.65	6
0.665	1.00			2.99	2.99	0
0.892	1.00			9.36	9.36	0
0.930	0.73	0.693	0.27	11.06	7.29	52
0.837	0.34	0.629	0.66	3.87	3.11	20
0.890	0.69	0.650	0.31	7.16	5.00	44
0.811	0.92	0.323	0.08	4.97	3.61	37
0.803	0.86	0.289	0.14	4.57	2.81	62
0.880	0.68	0.692	0.32	8.77	5.27	66
0.725	0.87	0.358	0.13	3.39	2.78	22
0.648	1.00			2.84	2.84	0
0.902	1.00			10.20	10.20	0
0.850	0.68	0.513	0.32	5.21	3.38	54
0.488	1.00			3.06	3.06	0
0.926	0.96	0.553	0.04	12.98	10.40	23
0.864	0.94	0.464	0.06	7.03	5.66	24
0.726	0.82	0.342	0.18	3.25	2.42	34
0.781	0.82	0.602	0.18	4.19	3.74	12
0.744	0.63	0.389	0.37	3.07	2.29	34
0.537	1.00			2.16	2.16	0
0.865	0.82			7.99	4.52	26
0.812	0.80			5.50	4.17	31
0.712	0.83			3.78	3.09	20

and 2 exercise experiments, as described in the text. The 1st column refers to the text. All volumes have been corrected to body temperature, ambient pressure,

F.R.A. is also the same during rest and exercise, i.e. 8.2, 8.0 and 8.2 liters respectively. Here the variability is smaller as shown by the standard deviations: ± 0.54, ± 0.50 and ± 0.80 liters.

Table II.

Work freq./min.	Breaths /min.	Tidal volume ml.	Wash-out time min.	F.R.A. L.	Vol. 0_2/ L. FRA.	w_1
rest.....	14.0	600	2.2	2.4	7.7	0.907
56.....	12.9	850	1.6	2.3	7.7	0.824
112.....	15.8	950	1.2	2.2	8.2	0.859
rest.....	14.7	580	2.3	2.4	8.2	0.886
56.....	13.1	890	1.5	2.2	8.0	0.818
112.....	15.0	800	1.2	2.0	7.2	0.840
rest.....	13.9	560	2.5	2.5	7.8	0.918
56.....	13.5	770	1.7	2.4	7.4	0.854
112.....	16.1	790	1.3	2.3	7.2	0.846
rest.....	12.6	671	2.2	2.2	8.5	0.866
56.....	12.8	814	1.8	2.3	8.1	0.831
112.....	13.8	1121	1.3	2.2	9.1	0.805
rest.....	11.0	703	2.5	2.4	9.1	0.860
56.....	11.7	915	1.8	2.3	8.4	0.805
112.....	10.0	1242	1.4	1.9	9.1	0.766
rest.....	10.8	656	2.6	2.2	8.4	0.858
56.....	10.0	945	1.9	2.1	8.6	0.799
112.....	12.1	1056	1.4	2.0	8.9	0.793
rest.....	10.8	658	2.5	2.1	8.6	0.870
56.....	11.2	876	1.7	1.9	9.0	0.797
112.....	13.1	977	1.3	1.8	9.0	0.765
rest.....	10.8	662	2.5	2.3	7.3	0.860
56.....	12.5	848	1.6	2.1	7.7	0.802
112.....	15.6	1001	0.9	2.0	7.8	0.781
rest.....	12.8	595	2.5	2.3	8.3	0.847
56.....	11.9	900	1.6	2.1	7.5	0.790
112.....	12.1	950	1.4	1.9	8.6	0.760
Mean values						
rest.....	12.4	632	2.4	2.3	8.2	0.875
56.....	12.2	868	1.7	2.2	8.0	0.813
112.....	13.7	987	1.3	2.0	8.2	0.802

Discussion.

Our conclusion is that hyperventilation during light and moderate degrees of exercises, in which the increase in respiratory minute volume is brought about chiefly by an increase in tidal volume, is

Subject G. L.

f_1	w_2	f_2	w_3	f_3	Average nr. of breaths N_2 molecules remain		
					actual	ideal	delay in %
0.50	0.805	0.40	0.430	0.10	7.71	4.60	84
0.88	0.433	0.12			5.21	3.90	33
0.46	0.644	0.54			4.78	3.69	30
0.84	0.653	0.16			7.93	5.17	29
0.75	0.573	0.25			4.71	3.83	23
0.56	0.659	0.44			4.79	3.98	27
0.45	0.820	0.42	0.549	0.13	8.11	5.21	55
0.66	0.639	0.34			5.46	4.29	27
0.67	0.664	0.33			5.33	4.37	22
0.67	0.705	0.33			6.12	5.13	19
0.85	0.673	0.15			5.49	5.13	7
0.73	0.539	0.27			4.36	3.46	25
0.85	0.614	0.15			6.46	5.29	22
0.82	0.571	0.18			4.62	4.08	13
0.80	0.335	0.20			3.72	2.57	45
0.83	0.670	0.17			6.37	5.50	16
0.82	0.515	0.18			4.45	3.65	22
0.85	0.441	0.15			4.37	3.43	27
0.65	0.713	0.35			6.12	5.23	19
0.94	0.088	0.06			4.70	2.18	115
0.93	0.129	0.07			4.50	2.32	94
0.85	0.593	0.15			6.44	5.18	24
0.88	0.493	0.12			4.69	3.96	19
0.84	0.254	0.16			4.05	2.92	38
1.00					6.54	6.54	0
1.00					4.76	4.76	0
1.00					4.17	4.17	0
	0.619						30
	0.443						29
	0.407						34

not relatively more efficient in ventilating the lungs, as might be thought from the relative decrease in dead space ventilation. On the contrary, the efficiency is much the same as during rest. This is shown

both by the pulmonary nitrogen clearance delay percentages and by the volume of oxygen used to wash out one liter of functional residual air. Of these two indices, the latter appears to be the more stable indicator of the ventilatory efficiency. The reason for the large variability of the former index is that the form of the wash-out curve is difficult to draw, particularly when the subject is breathing unevenly, and the solution of the original curve into components will then be difficult and rather subjective. This is particularly the case in experiment no. 7, Table II, where for work experiments the delay percentage is 115 % and 94 %, whereas all other values for this subject indicate normal ventilatory efficiency. As has been pointed out above, when w and f are both small the possible error in $\frac{1}{1-w}$ is large. The wash out volume per liter F.R.A. seems to be a value of better reproducibility than the clearance delay percentage. Here the values are chiefly dependent on a correct F.R.A. value. This is easily measured in trained subjects who can cooperate perfectly. When dealing with patients or untrained subjects there may be difficulties, chiefly due to changes in the respiratory level. Our experience indicates, however, that it is possible to obtain reproducible duplicate estimations of F.R.A. with our method in the large majority of subjects, but it may be difficult at times to choose the right moment to change from room air to oxygen breathing at the bottom of a normal expiration. The measuring error in the determination of the total amount of nitrogen expired during the wash-out period is much smaller, in our experiments in the order of ± 2 %.

The variability of all indices of the extent of unequal ventilation, even when using very rapid gas analysis, has to be stressed. This appears clearly from a study of Table II. The lung clearance index may range from 7.3 to 9.1 in the same individual, trained in respiratory experiments, at rest. In most experiments, this subject has a two-compartment ventilation, but in one experiment (no. 9) he is ventilating uniformly, and in two (nos. 1 and 3) a small third compartment separates out.

The nitrogen wash-out time is in itself not a valid indicator of the ventilatory efficiency, as the influence of the ventilatory minute volume is not taken into account and the volume to be washed out (F.R.A.) is also neglected.

These findings have to be taken into account in clinical studies

when the impairment of ventilatory efficiency is to be judged from a single or duplicate experiment. In such studies the method described has definitely the advantage of providing several indications of ventilary efficiency which are to some extent independent of one another.

Summary.

The ventilatory efficiency has been studied at rest and during exercise in 9 normal subjects. For light and moderate degrees of work the relative efficiency is the same as at rest. This conclusion is based upon determination of the pulmonary nitrogen clearance delay percentage (FOWLER) and of the lung clearance index (BECKLAKE); the latter is the more dependable of the two indices. The wash-out time, i.e. the time it takes to wash out the pulmonary nitrogen from 80 % down to 2 %, decreases with increasing respiratory minute volume and increases with increasing lung volume. For gas analysis a modified nitrogen meter (LUNDIN and ÅKESSON 1954) was used.

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Localization of Micro-Electrode Sites by Silver Impregnation.

By

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Introduction.

Micro-electrodes are coming more and more into use in neurophysiological research. It is well known that the exact localization presents difficulties. Many methods have been devised but none satisfies all requirements as they are either too complicated and difficult or too crude.

Well known are the methods depending on staining sections with carbo-thionin, cresyl-violet, or treatment according to the method of Weil, or impregnation with osmic acid. Some investigators have left the electrode in situ and made sections of 3—4 mm thickness pellucid by methyl salicylate. Others have identified the electrode track by electrolysis, or by deposition of iron with subsequent intravital perfusion of ferro-cyanide, producing Prussian blue.

In work with micro-electrodes in the hippocampus the author has made use of sections silver-impregnated by the method of NAUTA (1950), modified for paraffine sections by BLACKSTAD (1954). This method has some advantages: The high contrast between the electrode track and the background facilitates the identification of the track; the tissue is not destroyed; it allows quite a number of electrode tracks in the same tissue block; finally, it is possible to stain intervening sections according to other methods (for instance, when it is the the question of localizing the track in relation to nuclear masses).

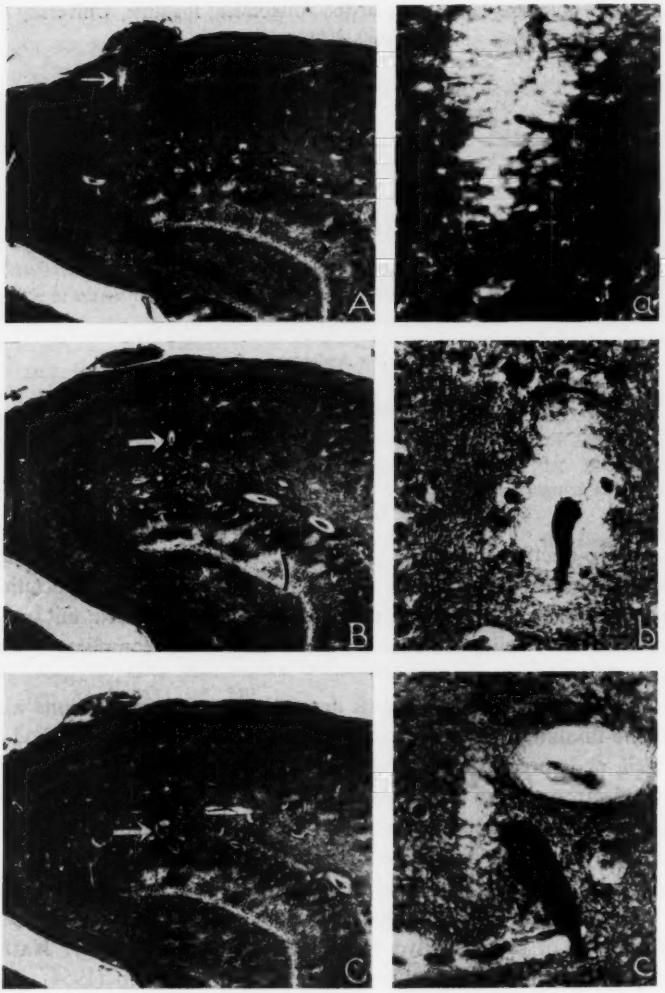
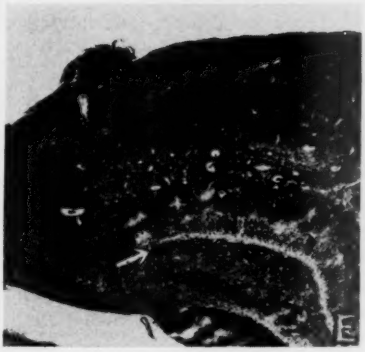


Fig. 1. *A*–*E* demonstrate one electrode track in the hippocampus of cat (white arrows); each 6th section is shown. Note the regular intervals between the defects. Compression artefacts are seen as "plow furrows" parallell to the upper surface (best seen in the lower halves of the pictures).

a–*e* show the electrode defects with greater magnification. Note the vacuolization in *a* and *c*, the black-stained central detritus in *b*, the great and the small capillary and the silver precipitate in *c*, and the small hemorrhage in *e*.



Procedure.

Sectioning and staining.

1. Withdraw the electrode, exsanguinate the brain and fix for at least a couple of days in 10 per cent formalin.
2. Isolate small blocks containing the electrode tracks and embed in paraffine in the usual way.
3. Cut sections of 15 μ thickness, mount every 3rd. It is important to cut the block as parallell to the electrode track as possible, and preferably in the direction in which the electrode was pushed into the tissue in order to avoid confusion with compression artefacts produced by the cutting. The electrode defects will then cross the compression artefacts at right angles and thus be more easily recognized. (Fig. 1 A—E.)

4. Dehydrate in graded alcohols.
5. Immerse in silver-pyridine solution for 2 hours at 54° C (1.5 g silver nitrate is dissolved in 50 ml distilled water; 2.5 ml pyridine is added). The solution must be warmed in a thermostat before use.
6. Without washing bring the sections for about half a minute into an ammoniacal silver solution, to be prepared as follows: 450 mg silver nitrate is dissolved in 33 ml distilled water. 15 ml 100 per cent ethanol is added. The mixture is allowed to cool to room temperature. 2 ml 25 per cent ammonia, and 1.25 ml 4 per cent solution of sodium hydroxide are added. The solution contains ammonia in excess and should be covered to avoid evaporation.
7. From the silver-ammonia bath transfer the sections quickly to the reducing solution, composed of:

distilled water	45 ml
100 per cent ethanol	5 ml
10 per cent formalin	1.5 ml
1 per cent citric acid	1.5 ml

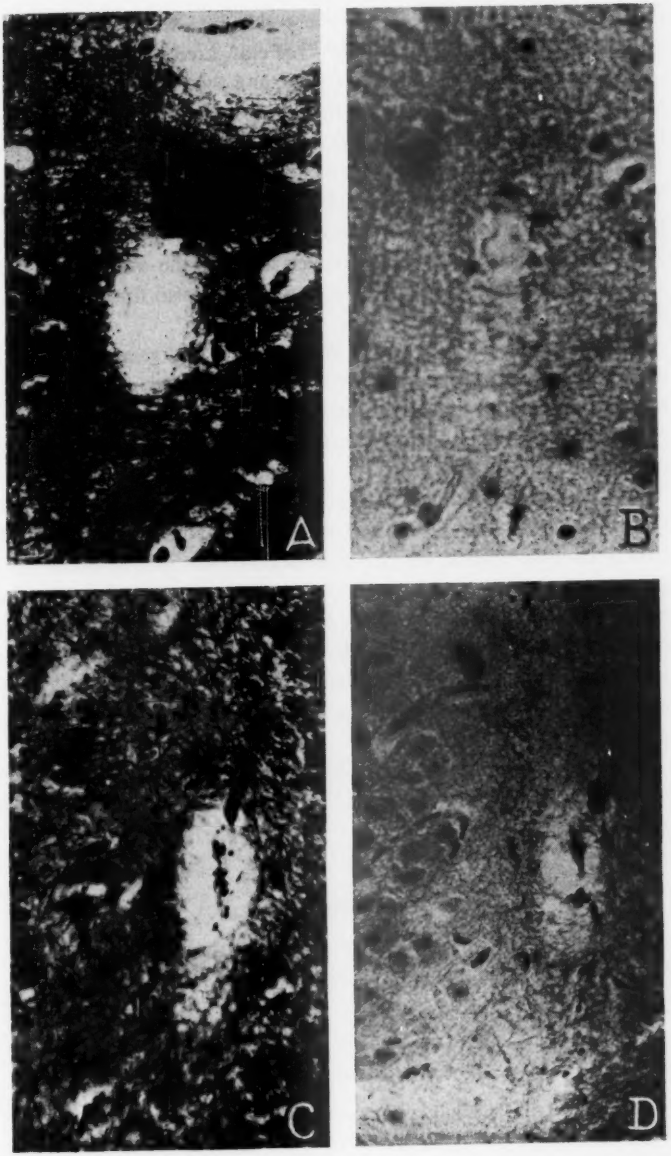
   The sections remain in this solution for about 1 minute until they have obtained a brown colour. The reducing solution should be used only once.
8. The reducing process is interrupted by immersion in ample amount of distilled water.
9. Immerse for 2 minutes in citric acid solution consisting of 1 part of 1 per cent citric acid and 20 parts of distilled water.
10. Wash thoroughly in two changes of distilled water.
11. Dehydration in graded alcohols and three changes of xylene.
12. Canada-balsam.

Microscopical technique.

When the sections are examined under low power, the electrode tracks stand out as white specks against the yellow or light brown background (Fig. 1 A—E). It is, therefore, better to impregnate the sections too heavily than too lightly. To be regarded as an electrode track, the defect must be present in adjoining sections, changing site with regular intervals (Fig. 1 A—E).

Fig. 2. Comparison of various staining methods.
A—B. Adjoining sections of one electrode track stained according to the modified Nauta method (A) and with thionin (B).

C—D. Adjoining sections of another electrode track treated according to the modified Nauta method (C) and stained after the method of Woelcke (D).


A
B
C
D

Certain characteristics of the micro-electrode track may be mentioned. The most constant feature is the elongated shape (Fig. 1 a—e) indicating the direction of the electrode, and the vacuolization around the track (Fig. 1 a and c). Other indications of an electrode track are bleeding outside a vessel (Fig. 1 e), systematic deviation of fibers (Fig. 1 e) and elongation and pycnosis of nerve cell nuclei (Fig. 1 a and d). The track is always greater than the electrode due to the shrinkage of the tissue. With some experience it is possible to identify all tracks. The smallest electrode shaft diameter used by the author has been 15 μ, but undoubtedly the method can be used also for electrodes of smaller diameter.

One may be deceived by other defects in the background, especially by capillaries. The latter are more regularly bounded, not surrounded by vacuoles and are not as straight as the electrode tracks (Fig. 1 c). Failure of impregnation and knife artefacts are usually readily recognized.

The described modified Nauta method has been compared with the method of BODIAN (1937) and with those of NISSL and of WOELCKE (1942) (myelin sheath stain). The Bodian method is more time-consuming and the photographical contrast is somewhat smaller, but it will as a rule give good results. However, insufficient impregnation is more likely to occur with the Bodian technique than with the modified Nauta method. The methods of Nissl and of Woelcke do not by far demonstrate the track as clearly as does the modified Nauta method (Fig. 2).

Toning of Nauta preparations with gold has also given satisfactory results. The procedure has been as in the Bodian method (BODIAN 1937).

Summary.

A silver impregnation method for demonstrating the site of micro-electrodes in the central nervous system is described.

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The Effect of Hypothalamic Lesions on the Water Intake of the Dog.

By

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The extent of the area of the hypothalamus that produces polydipsia when stimulated electrically has been reported recently (ANDERSSON and MCCANN 1955 c). To determine if this region is really essential for the regulation of water intake, attempts to destroy it were made in dogs, and the water and electrolyte metabolism of the animals was studied. This seemed of particular interest since STEVENSON and his collaborators (STEVENSON 1949, STEVENSON, WELT and ORLOFF 1950) and WITT, KELLER, BATSEL and LYNCH (1952) have reported hypodipsia and adipsia following lesions associated with hypothalamic obesity and following anterior hypothalamectomy, respectively.

A brief preliminary report of the present work has already appeared (ANDERSSON and MCCANN 1955 b).

Methods.

Hypothalamic coagulations were carried out on 22 occasions in 15 dogs of assorted breeds. Operations were made under morphine-nembutal anaesthesia, using Hess' technique for electrocoagulations (1954), with the placement of the electrodes controlled by prior X-ray. The electrode holder was either placed on the dorsum of the skull, if this was flat, or the frontal sinus was opened and the holder placed on the floor of the sinus. Both

before and after the operations, the animals were kept in metabolism cages. They were fed a constant diet (in most cases boiled horse meat, 25 grams per kilogram body weight per day). A constant amount of sodium chloride was added to the daily food ration of each dog to make it contain approximately 2 mE./kg. body weight/day. Water intake, urine volume, and urinary sodium, chloride and potassium outputs were measured during a control period and after the hypothalamic lesions had been placed. Plasma sodium and chloride concentrations were determined at appropriate intervals.

Chemical methods: Sodium and potassium analyses in plasma and urine were made using the EEL flame photometer with external standards. Plasma chloride determinations were made by the method of VAN SLYKE (1923) and urine chloride according to the Volhard method (PETERS and VAN SLYKE 1932). Blood non protein nitrogen was determined occasionally according to FOLIN (1930).

Histological technique: At the termination of an experiment the dog was killed by intravenous nembutal, and the head was perfused with Bouin's fixative. After fixation in Bouin's and paraffin imbedding, the hypothalamus with attached pituitary was serially sectioned in horizontal sections and stained by Gomori's chrom alum hematoxylin method (GOMORI 1941) as described by BARGMANN (1950).

Autopsy: Animals which died before the end of an experiment were carefully autopsied at the Dept. of Pathology of this college after removal of the head for examination of the hypothalamic lesions.

Results.

During the control periods all animals drank between 20 to 25 ml. of water/kg. of body weight/day. In evaluating the effect of the hypothalamic lesions on water intake, only animals which were ambulatory and eating normally after the operation were considered. With these criteria, the water intake was significantly reduced in 6 animals with hypothalamic lesions. In the three cases with the most striking changes, the dogs refused to drink water for periods of 9 to 14 days postoperatively in spite of progressive dehydration up to the point where fluid had to be administered to maintain the animals. During this self imposed water restriction, dehydration was evidenced by loss of tissue turger, by weight loss of up to 12 % of body weight in spite of normal food intake, and by striking elevations of plasma sodium and chloride concentrations. During the dehydration, urine volume became reduced to a constant low figure as the specific gravity climbed to a maximum value for each animal. After significant dehydration was present, there was a failure to respond with water diuresis to water loads of up to

10 % of the body weight. Even during the periods of adipsia, all of the 6 dogs readily drank milk or broth, when this was offered to help restore fluid loss. Water metabolism was studied for periods ranging from 9 to 50 days after production of hypothalamic lesions, and during this time, 4 of the 6 animals exhibited a partial recovery of drinking.

To determine whether or not alterations in the excretion of electrolytes might play a role in the development of the high plasma electrolyte concentrations frequently seen after these lesions, the urinary excretion of sodium, chloride, and potassium was determined while the dogs were on a constant intake of these electrolytes. In 2 animals the salt content of the diet was raised to 2 and 3 times the basal level for periods of 5 days each. With the exception of one dog which showed a significant retention of sodium and chloride throughout the postoperative period of three weeks, no definite changes in sodium, chloride or potassium balances were seen, even when the sodium chloride intake was increased.

Graphs of the water intake, urinary output, and plasma sodium and chloride concentrations of two of the three animals which showed the greatest impairment of drinking are given in Figs. 1 ("Beta") and 2 ("Sessan"). Supplementary fluid in the form of milk or broth was given at appropriate intervals to maintain the animals as shown on the graphs. As the electrolyte balances in these cases showed no definite abnormalities, they are not included in the charts. The third dog did not drink throughout the postoperative period and died with signs of marked dehydration on the 9th day in spite of attempts at rehydration on the 8th postoperative day. Except for the dog which exhibited a retention of sodium chloride as well as decreased water intake, the postoperative water and electrolyte metabolism of the other hypodipsic animals was similar to that shown on the graphs, but the defect in drinking was not quite so marked.

All hypodipsic dogs had hypothalamic lesions which to varying degrees bilaterally involved the area found previously to evoke drinking when stimulated electrically in the goat (ANDERSSON and MCCANN 1955 c). The degree of hypodipsia seemed to be roughly proportional to the extent of the damage to the "drinking area". Thus, in "Beta" and "Sessan", whose postoperative course has been illustrated (Figs. 1 and 2), most of this area was destroyed (Fig. 3). The lesion in the exceptional animal which showed a significant retention of sodium and chloride in addition to impaired water intake lay more rostrally

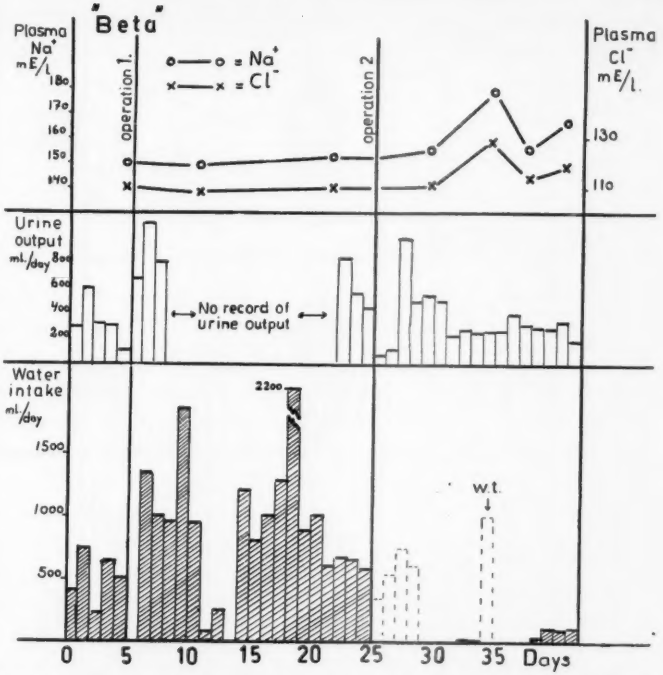

Fig. 1. A graph of the changes in water metabolism and plasma sodium and chloride concentrations in the dog "Beta" (preoperative weight: 14.8 kg.) after electrocoagulations were made on two occasions in the hypothalamus. The first coagulation (operation 1) caused diabetes insipidus but no impairment of drinking was observed, whereas the second (operation 2) was followed by temporary adipsia.

The extent of the final lesion in the hypothalamus of this animal is shown in Fig. 3. Dotted columns on the graph of water intake mark the amount of milk given as supplementary fluid and water given by stomach tube (w.t.).

than in the other animals and involved only the rostral portion of this "drinking area".

Diabetes insipidus of mild degree was seen after the operation in 2 of the 6 dogs with effective lesions. It was absent in the others, including 2 of the 3 animals which exhibited the most prolonged period of adipsia. In fact serial sections revealed that the supraopticophypophyseal system in "Sessan" was completely intact.

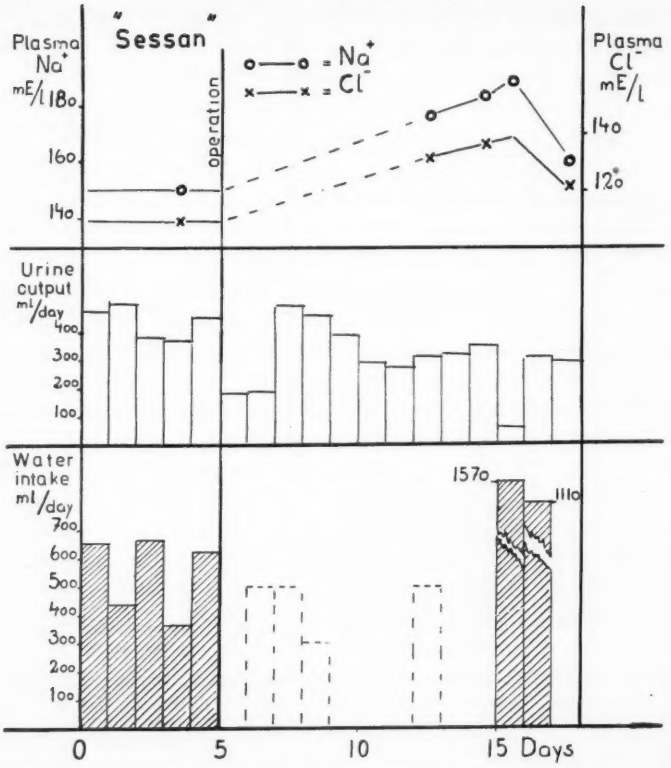

Fig. 2. A graph of the changes in water metabolism and plasma sodium and chloride concentrations in the dog "Sessan" (preoperative weight: 24.5 kg.) after a lesion in the hypothalamus. The extent of the hypothalamic lesion of this dog is shown in Fig. 3.
Dotted columns on the graph of water intake indicate the amount of milk and broth given as supplementary fluid.

In contrast to the above findings, two additional dogs did not show impaired drinking after operation but developed diabetes insipidus after damage to the supraopticohypophyseal system. They drank large volumes of water and did not become significantly dehydrated as evidenced by maintainance of body weight and normal values for plasma electrolytes.

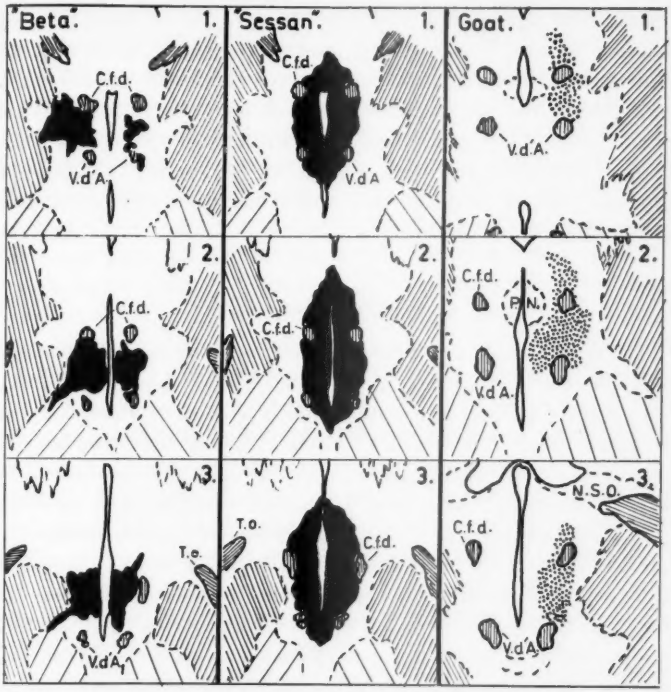

Fig. 3. Diagrams of horizontal sections through the dorsal (1), middle (2) and ventral (3) hypothalamus of the dogs "Beta" and "Sessan", whose postoperative water intake is shown in Fig. 1 and 2, and of a goat. The black areas mark the extent of the hypothalamic lesions which caused temporary adipsia and the dotted areas on the sections of the goat mark the region of the hypothalamus where electrical stimulation produced polydipsia (ANDERSSON and MCCANN 1955 c).
C.f.d. = Columna fornicis descendens.
N.S.O. = Nucleus supraopticus.
P.N. = Nucleus paraventricularis.
T.o. = Tractus opticus.
V.d'A. = Tractus Vicq d'Azyr.

The mortality from the hypothalamic operations was 37 % and occurred predominantly in the animals where the coagulation had produced the largest lesions. Death usually occurred within the first 24 hours, and the autopsy diagnosis was hemorrhagic diathesis, with bleedings seen especially in the gastrointestinal tract, and subendo- and epicardially. After having shown a brief recovery, two animals

deteriorated rapidly and died after 4 days, one of them showing evidence of renal shutdown and uremia. The urine contained much albumin and blood, and the blood non protein nitrogen was 106 mg.%.

Studies of the neurosecretory material in the hypothalamus and neurohypophysis failed to reveal any consistent changes, except where parts of this system had been damaged by the lesions. This might be explained by the fact that all animals had been at least partially rehydrated prior to death.

Discussion.

The three dogs in which the "drinking area" was largely destroyed all showed at least a temporary adipsia. One of them, "Lisa", died with signs of extreme dehydration, nine days after the operation, and both the others, "Sessan" and "Beta", rapidly developed marked dehydration and had to be given supplementary fluid in order to be kept in condition to eat and to drink milk. However, both showed a partial recovery after 11 and 14 days, respectively. Since during these periods of adipsia, the "thirst stimulus" certainly must have been extremely strong, one would expect that the hypothalamic lesions had temporarily completely inactivated the "drinking area", but that after a time some of it might have regained its function. The animals became dehydrated more rapidly when not drinking than was the case in earlier experiments where dogs were allowed neither food nor water (GAMBLE, ROSS and TISDALL 1923, ELKINTON and TAFFEL 1942). This was probably due to the high protein diet with added sodium chloride fed the present dogs, which necessitated a comparatively large urine volume.

With the exception of the animal with a more rostrally placed lesion which retained significant amounts of sodium chloride, no net retention or excretion of sodium chloride was observed which makes it probable that most of the animals had no primary disturbance in sodium chloride metabolism. Further evidence for this was provided by the fact that 2 of the dogs with impaired drinking disposed of additional loads of sodium chloride without difficulty. The failure of the dogs to develop a negative sodium chloride balance in the face of high plasma sodium and chloride levels during dehydration is similar to the response of normal dogs which do not excrete appreciable sodium or chloride in spite of high plasma levels when both food

and water are withheld (ELKINTON and TAFFEL). When the animals were dehydrated, water loading did not result in water diuresis which might be expected because of the hypertonicity of the body fluids. This presumably would serve as a continued stimulus for the secretion of antidiuretic hormone by the neurohypophysis (VERNEY 1947).

STEVENSON and his collaborators (STEVENSON 1949, STEVENSON, WELT and ORLOFF 1950) have previously reported a slightly decreased ratio of water to food intake, a decreased response to water loads, and moderately elevated plasma sodium concentrations in rats with hypothalamic obesity. These findings were interpreted as indicating the existence of a state of "hypothalamic hypodipsia". It is felt that histological examination of these lesions in the rats with "hypothalamic hypodipsia" might demonstrate that they involved the "drinking area" but did not completely destroy it, thus accounting for the relatively small changes found in water metabolism in these cases.

The lesions in the anterior hypothalamectomies which WITT, KELLER, BATSEL and LYNCH reported to have occasionally led to permanent adipsia may have been of such an extent as to involve the *entire* "drinking area". This interpretation of their findings would be consistent with the results reported here and with the preceding stimulation experiments (ANDERSSON 1952, ANDERSSON and MCCANN 1955 a and 1955 c).

The present results show that the involvement of the supraoptico-hypophyseal system present in the hypothalamectomies of WITT *et al.* is not an essential condition to development of adipsia. In fact, the presence of adipsia together with maximum urine concentrations in the present cases suggests that the regulation of antidiuretic hormone secretion for water conservation is at least partially independent of the regulation of water intake.

A number of clinical cases have been reported where injury to the basal portion of the brain in man was followed by elevations of plasma sodium and chloride concentrations. This finding has been interpreted by some workers to be the consequence of damage to either a "drinking center" (ENGSTRÖM and LIEBMAN 1953, ELLIOT 1955, and ROTHBALLER and DUGGAR 1955), or a center regulating electrolyte metabolism (WELT, *et al.* 1952, COOPER 1953). The data reported here give as yet no conclusive evidence for the later explanation. However, strong evidence is now available indicating that an essential drinking center is located in the hypothalamus, since stimulation of a well

defined part of the hypothalamus produces drinking, whereas destructive lesions of this same area can result in a marked decrease in water intake. The present results also suggest that complete destruction of this part of the hypothalamus would give a permanent adipsia.

Summary.

Hypothalamic lesions which caused hypodipsia or adipsia lasting up to 14 days after the operation were made in dogs. The animals developed marked dehydration as a consequence of the impaired water intake. In contrast to the absence of "thirst", the dogs ate and drank milk and broth readily. No correlation was found between the absence of drinking and the presence of diabetes insipidus. The degree of hypodipsia was roughly proportional to the extent of the destruction of the same area, which on electrical stimulation evokes drinking in the goat.

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The Inhibition of Hexokinase Reaction in the Rat Diaphragm by 2,4-Dinitrophenol.

By

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Received 12 September 1955.

The effect of 2,4-dinitrophenol (2,4-DNP) on the muscle metabolism in vitro was first demonstrated by RONZONI and EHRENFEST (1936). In isolated frog muscles 2,4-DNP produced an increased O_2-consumption, breakdown of glycogen and increased production of lactic acid. Furthermore the concentration of phosphocreatine was reduced while the content of ATP was unchanged.

Similar effects of 2,4-DNP in vitro on rat diaphragm have been demonstrated. Thus, VILLE, DEANE and HASTINGS (1949) found that 2,4-DNP increased the conversion of C^{14}-labelled glucose to CO_2. SACKS and SINEX (1952) showed that the concentration of ATP and phosphocreatine was reduced by 2,4-DNP while inorganic phosphate was increased. PIERCE and FIELD (1949) showed that 2,4-DNP produced a breakdown of glycogen in the diaphragm, but found no definite effect on the glucose uptake. TERNER (1951), however, found that 2,4-DNP gave inhibition of the glucose uptake in mammary gland slices.

In the present work it has been demonstrated that 2,4-DNP inhibits the glucose uptake in the diaphragm both under aerobic and anaerobic conditions. The effect of 2,4-DNP on the hexokinase reaction is further examined in the particulate and soluble fraction of homo-

genate of rat diaphragm. The distribution and properties of the hexokinase in rat diaphragm has been investigated, and it is shown that 2,4-DNP inhibits the hexokinase reaction in both fractions.

Methods.

Experiments on the isolated rat diaphragms.

Young male rats weighing 100–140 g, which had fasted for 24 hours were used. Incubation of the removed diaphragm was carried out in saline-phosphate medium, pH 7.4. Initially the medium contained 140 mg glucose per 100 ml. Incubation took place for 60 mins. at 37° C. 100 per cent oxygen or 100 per cent nitrogen was used as the gas phase. The procedure is otherwise described previously (WALAAS and WALAAS 1950, 1952). Glucose was determined by the method of NELSON (1944) and glycogen according to WALAAS and WALAAS (1950). The lactic acid determination was carried out according to BARKER and SUMMERSON's (1941) method. The determination of the phosphorus containing compounds in the diaphragm is described previously (WALAAS and WALAAS 1952). For the determination of inorganic phosphate, the method of LOWRY and LOPEZ (1946) was used, and that of FISKE and SUBBAROW (1925) for the determination of phosphocreatine and ATP.

Experiments Carried out on Fractions of the Diaphragm.

Adult rats weighing 250–300 gr. were used after fasting for 24 hours. Diaphragms from 15–20 rats were employed in each experiment. Homogenization was carried out in 0.25 M sucrose or 0.154 M KCl, to which was added 0.01 M Trisbuffer pH: 8.0, in an all glass grinder for 15 minutes under cooling. Differential centrifugation was carried out according to SCHNEIDER and HOGEBOOM (1950). The nuclear fraction was removed by centrifugation at 600 × g for 10 mins. The particulate fraction was separated from the supernatant by centrifugation at 15 000 × g for 20 minutes. The centrifugation was carried out in an International refridgerated centrifuge. The procedure is described in detail in a subsequent paper (WALAAS, BRIGHAM and BRINCK-JOHNSEN 1956).

Procedure for the Determination of Hexokinase.

The method described by COLOWICK, CORI and SLEIN (1947) was used with a few modifications. The incubation medium contained (concentrations): 0.001 M glucose, 0.002 M ATP, 0.01 MgCl, 0.053 M KF, 0.03 M tris (hydroxymethyl)aminomethane buffer, pH: 8.2. 0.5 ml of the enzyme solution, equivalent to 50 mg of tissue was added. Total volume in each flask was 3.0 ml. Equilibration was carried out with 100 % N_2. When homogenization was carried out in 0.154 KCl, bicarbonate buffer 0.025 M $KHCO_3$ was used employing a gas phase 95 % N_2/5 % CO_2.

Incubation was carried out for 20 minutes at 30° C. The reaction was stopped by the addition of 1.0 ml 0.3 N $Ba(OH)_2$ + 1.0 ml 0.3 N $Zn\,SO_4$ and glucose analyzed according to the method of NELSON. In those experiments where glucose-6-phosphate was used, the addition of 3.0 ml $Ba(OH)_2$ + 3.0 ml $Zn\,SO_4$ was required.

Materials.

The Ba-salt of ATP from Sigma Chemical Company was used after conversion to the K-ATP by means of an ion exchange resin (Amberlite 120) according to KIELLEY and MEYERHOF (1948). The Ba-salt of glucose-6-phosphate from Sigma Chemical Company was converted to the K-salt by the method of COLOWICK and SUTHERLAND (1942). 2,4-dinitrophenol (Na-salt) was a pure Merck product.

Characterization of Rat Diaphragm Hexokinase.

pH during Homogenization.

From Table I it is seen that the hexokinase activity was considerably higher when the homogenization was carried out at pH 8.0 than at pH 7.0. This was the case when both isotonic sucrose or isotonic

Table I.

The effect of pH during homogenization on hexokinase activity in homogenate and soluble fraction of rat diaphragm.

Homogenization Medium	pH in homogenate	Hexokinase activity		
		Diaphragm homogenate	Soluble fraction of diaphragm	
		Glucose phosphorylation	Glucose phosphorylation	Glucose phosphorylation
		μg per 50 mg tissue equivalent		μg per mg nitrogen
0.154 M KCl	7.0	272	215	406
0.154 M KCl + 0.15 M $KHCO_3$	8.0	420	328	426
0.25 M sucrose	7.0	239	148	200
0.25 M sucrose + 0.01 M "Tris" buffer	8.0	390	358	510

In these experiments paired hemidiaphragms from the same rats were homogenized with and without buffer.

The pH in the incubation mixture was always 8.2.

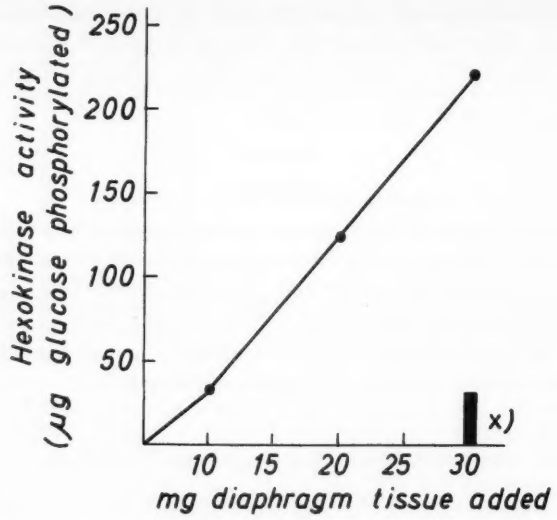

Diagram I.
The effect of enzyme concentration on the hexokinase activity in rat diaphragm homogenate.
Homogenates in different amounts added to the system.
Incubation for 10 mins at 30° C.

x) Represents glucose uptake of the isolated rat diaphragm.
Incubation in Krebs-Ringer phosphate medium, pH : 7,4.
Glucose 0.0078 M, (140 mg per 100 ml).
Incubation in 100 % O_2 at 30° C. Calculated for 10 mins.

KCl was used as medium during the homogenization. Thus a partial irreversible inactivation of the hexokinase occurs during homogenization at pH 7.0. Previously an inactivation of muscle hexokinase at pH 6.0 is described by COLOWICK and PRICE (1945).

As a result of this observation the pH in the homogenization medium was always adjusted to 8.0 by the addition of buffer.

Enzyme Concentration.

The phosphorylation of glucose in homogenate of rat diaphragm increases linearally with the amount of homogenate added (Diagram 1). From the diagram it is further seen that at a glucose concentration of 140 mg per 100 ml medium the glucose uptake in the surviving

Table II.

Carbohydrate balance in experiments on the soluble fraction of the rat diaphragm.

Glucose phosphorylation $M \times 10^{-3}$	Reaction products formed				
	Hexose-monophosphate[1]		Fructose-1-6-diphosphate[2]		Total recovery
	$M \times 10^{-3}$	%	$M \times 10^{-3}$	%	%
1.28 (8)	0.94 (8)	73[3]	0.23 (8)	18	92

Incubation at 30° for 20 mins.
Mean values are given.
Number of experiments in ( ).

[1] Determined as transphosphorylation of ATP to acid stable organic P.
[2] Determined as inorganic P liberated between 10 mins and 30 mins hydrolysis in N H_2SO_4.
[3] Simular values were observed by determination of hexosemonophosphate enzymatically with Warburg's Zwischenferment.

diaphragm is only approximately $^1/_{10}$ of the phosphorylation of glucose in homogenate of the diaphragm. Thus the cellular structure is of decisive importance for the regulation of the velocity of the phosphorylation of glucose in muscle.

Time of Incubation.

From Table II it is seen that 73 % of the phosphorylated glucose was converted to hexosemonophosphate in experiments with the soluble fraction of the diaphragm, while a smaller portion was converted further to fructose-1-6-diphosphate. CRANE and SOLS (1953) showed an accumulation of hexose monophosphate as a product in the hexokinase reaction in brain particles, while WEIL-MALHERBE and BONE (1951) found quantitative conversion of glucose to fructose-1-6-diphosphate in brain extract. The phosphorylation of glucose in homogenate of the diaphragm exhibits a gradually diminishing velocity after incubation for 15 minutes (Diagram II). The reason for this apparent reversibility of the hexokinase reaction must be sought in the fact that glucose-6-phosphate is an effective inhibitor for the hexokinase in the diaphragm. CRANE and SOLS (1953) have shown the same phenomenon in brain particles.

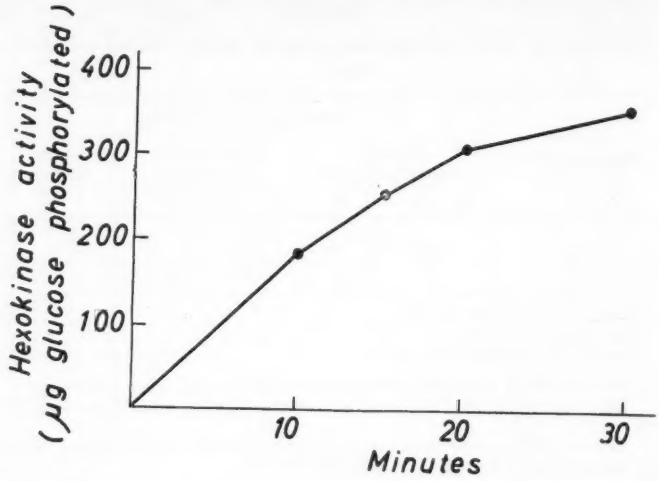

Diagram II.
The effect of time of incubation on hexokinase activity in rat diaphragm homogenate.

The Effect of pH.

As shown in diagram III the optimal activity for the particulate hexokinase in the diaphragm is at pH 8.0. In the soluble fraction the optimal activity was found at pH 8.2 and the curve is more irregular. Similar pH activity is given by COLOWICK (1951) for the muscle hexokinase and by SALTMAN (1953) for plant hexokinase. The activity of brain hexokinase is at a maximum at pH 6 to 8 (SOLS and CRANE 1954).

The Affinity of Hexokinase to ATP.

In the soluble fraction of the diaphragm K_S for ATP was 1.7×10^{-3} M and 2.4×10^{-3} M when homogenized in isotonic KCl and isotonic sucrose respectively. Values of the same order of magnitude were found by SALTMAN (1953) for plant hexokinase.

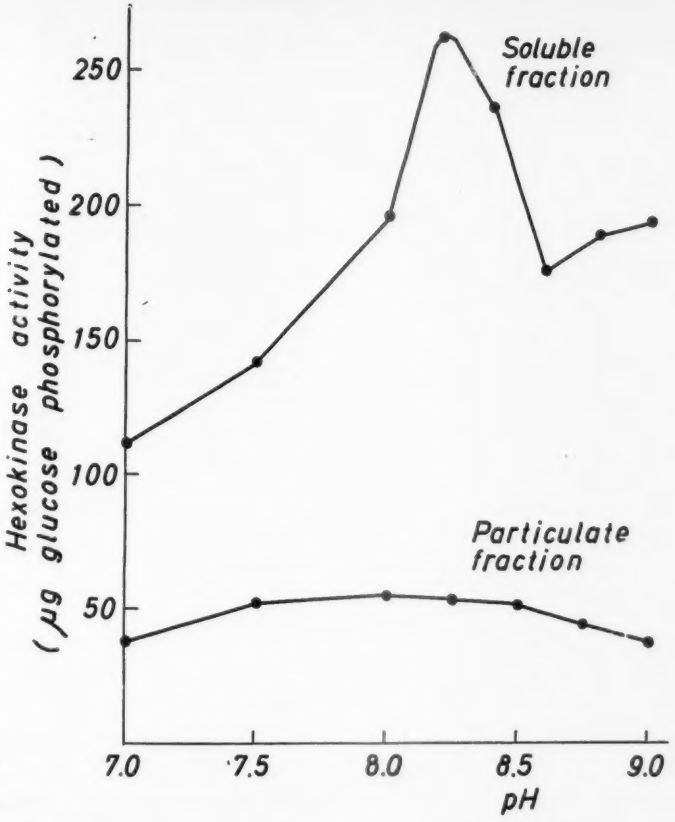

Diagram III.
The Effect of pH on hexokinase reaction of soluble and particulate fractions of the rat diaphragm.

Results.

Experiments with the Isolated Diaphragm.

From Table III it is seen that 2,4-DNP in a concentration of 5×10^{-5} M inhibited the uptake of glucose in the diaphragm, a statistically significant effect (Table VI). Simultaneously an almost complete breakdown of glycogen occurred while the production of lactic acid was slightly increased. At the same time (Table IV) a

Table III.

The effect of 2,4-DNP on glucose uptake, glycogen synthesis and lactic acid production of rat diaphragm under aerobic conditions.

Group	Glucose uptake	Glycogen			Lactic acid production
		Initial value	Final value	Synthesis	
	mg per g wet tissue per hour	mg per g wet tissue		mg per g wet tissue p hour	
Controls.....	1.7 ± 0.21(4)	2.4	4.0	+1.6±0.21(4)	0.58 ± 0.05(4)
2,4-DNP.....	1.0 ± 0.11(4)	2.4	0.6	−1.8±0.24(4)	0.86 ± 0.06(4)

2,4-DNP added to final concentration of 5×10^{-5}M, 100 % O_2.
Number of experiments in ( ).
Test of significance of the difference, controls versus 2,4-DNP:
Glycogen synthesis, t : 9,5, DF : 6, $P < 0.001$
Lactic acid production, t : 3.4, DF : 6, P : 0.01.

Table IV.

The effect of 2,4-dinitrophenol on the content of phosphorus compounds of the isolated rat diaphragm.

Group	Inorganic P	Phosphocreatine PC	ATP Labile P
	mg P per g wet tissue		
Controls.....	0.32 ± 0.01 (5)	0.23 ± 0.01 (5)	0.20 ± 0.01 (5)
2,4-DNP.....	0.50 ± 0.01 (5)	0.02 ± 0.01 (5)	0.05 ± 0.01 (5)
Insulin.....	0.30 (2)	0.25 (2)	0.19 (2)
Insulin + 2,4-DNP....	0.43 (3)	0.02 (3)	0.05 (3)

9.2 μg 2,4-DNP was added to 1 ml. incubation medium (5×10^{-5}M 2,4-DNP).
0.1 unit Insulin was added to 1 ml medium. Gas phase 100 % O_2.
Number of experiments in ( ).
Test of significance of the difference, controls versus 2,4-DNP:
Inorganic P, t:10, DF : 8, $P < 0.001$
PC, t:13, DF : 8, $P < 0.001$
ATP, t:9, DF : 8, $P < 0.001$

breakdown of phosphocreatine and ATP took place, while inorganic P increased. The inhibition of the glucose uptake by 2,4-DNP could not however be exclusively ascribed to the uncoupling of oxidative phosphorylations in the diaphragm. Thus Table V shows that 2,4-DNP gave inhibition of the glucose uptake even under anaerobic conditions and the effect was in this case stronger than under aerobic conditions.

Table V.

The effect of 2,4-DNP on glucose uptake and lactic acid production of the rat diaphragm under anaerobic conditions. 2,4-DNP added to final concentration of 5×10^{-5} M. 100 % N_2.

Group	Glucose uptake	Lactic acid production
	mg per g wet tissue per hour	
Controls.....	2.15 ± 0.09 (6)	3.55 ± 0.17 (4)
2,4-DNP.....	0.8 ± 0.07 (6)	2.5 ± 0.11 (4)

Number of experiments in ( ).
Test of significance of the difference, controls versus 2,4-DNP:
Glucose uptake t: 10,4, DF : 10, P < 0.001.
Lactic acid production, t: 4,8 DF : 6, P : 0.003.

As shown previously the low ATP concentration in the diaphragm under anaerobiosis is no limiting factor for the glucose uptake (WALAAS and WALAAS, 1952). The anaerobic inhibition of the glucose uptake by 2,4-DNP is further reflected in a reduced lactic acid production. The experiments thus indicate a primary inhibition of the hexokinase reaction in the diaphragm in the presence of 2,4-DNP.

Furthermore it has been demonstrated that the hormonal effects on the rat diaphragm is influenced by 2,4-DNP. Thus 2,4-DNP abolished the stimulating effect of insulin on the glucose uptake (Table VI). In this case the diaphragm is pre-exposed to insulin and 2,4-DNP added during the final incubation. From Table VI it is further seen that 2,4-DNP and adrenaline additively inhibit the glucose uptake in the diaphragm. The fact that adrenaline alone inhibits the uptake of glucose in the diaphragm is previously shown (WALAAS and WALAAS 1950). Thus the value which was found on the addition of 5×10^{-5} M 2,4-DNP was reduced further by 30 % on the addition of 1.1×10^{-5} M adrenaline.

Experiments on the Soluble and Particulate Fraction of Rat Diaphragm. Distribution of Hexokinase.

As appears from Table VII 82 % of the hexokinase was found in the soluble fraction and 10 % in the particulate. A small amount was found in the nuclear fraction, probably due to particulate contaminations which were not removed during the washing procedure. This

Table VI.

The influence of 2,4-dinitrophenol on glucose uptake on the rat diaphragm exposed to insulin and adrenaline in vitro. 2,4-DNP added to final concentration of 5×10^{-5} M. Gas phase 100 % O_2. In group II the diaphragms were preexposed for 5 min. to ordinary medium containing 1 unit insulin per ml at 25° C. The diaphragms were washed twice in 25 ml medium. Final incubation with and without 2,4-DNP.

Group	Experimental	Glucose uptake mg per g wet tissue per hour
I	Controls	1.85 ± 0.12 (10)
	2,4-DNP	1.25 ± 0.10 (10)
II	Insulin	3.4 ± 0.12 (8)
	Insulin + 2,4-DNP	1.8 ± 0.12 (8)
III	2,4-DNP	1.9 ± 0.07 (5)
	Insulin + 2,4-DNP	2.2 ± 0.11 (5)
IV	2,4-DNP	1.6 ± 0.10 (5)
	Adrenaline + 2,4-DNP	1.1 ± 0.10 (5)

In group III the diaphragms were pre-incubated for 5 min. in medium with and without the addition of 1 unit insulin per ml. at 25° C. After washing twice in 25 ml medium the final incubation was performed in medium containing 2,4-DNP. In group IV 2,4-DNP was added to all flasks with or without 1.1×10^{-5} M adrenaline (2 µgm per ml.).

Number of experiments in ( ).

Test of significance of difference:

Group I, t:4,1, DF:18, P : 0.001.
Group II, t:9,4, DF:14, P<0.001.
Group III, t:2,2, DF:8, P : 0.06.
Group IV, t:3,5, DF:8, P : 0.007.

Table VII.

Distribution of hexokinase activity in the soluble and the particulate fraction of rat diaphragm. Mean values.

Fraction	Number of experiments	Hexokinase activity in % Mean values
Homogenate	9	100
Soluble	8	82
Particulate	6	10

corresponds to the conditions in liver where glucokinase, galactokinase and glycolytic enzymes are easily solubilized by homogenization and differential centrifugation (LE PAGE and SCHNEIDER 1948, HERS, BERTHET, BERTHET and DE DUVE 1951). On the other hand

CRANE and SOLS (1953) found 95 % of brain hexokinase associated with particles by centrifugation at 8000 × g. In plants SALTMAN (1953) demonstrated great differences, from 100 % particle bound hexokinase in potatoes to 19 % in seed.

It is possible that the demonstrated distribution of hexokinase in the diaphragm does not present a true picture of the situation in vivo. The possibility that part of the hexokinase is eluted from the particles must be taken into consideration. As demonstrated in the following paper the particles isolated from the diaphragm posess ATP-ase activity. This indicates "ageing" of the particles during the long period of homogenization required.

The Effect of Glucose-6-phospahte.

In experiments with homogenate of the diaphragm, it was shown that G-6-P in a concentration up to 3×10^{-3} M was not dephosphorylated. Therefore the diaphragm was without glucose-6-phosphatase activity.

As a result of this observation the hexokinase activity may be measured without correction for the liberation of glucose.

In Table VIII it is shown that G-6-P is a strong inhibitor for the hexokinase of the diaphragm. The inhibitory effect of G-6-P on brain hexokinase is shown by WEIL-MALHERBE and BONE (1951), and by CRANE and SOLS (1953), while yeast hexokinase is not affected by G-6-P (COLOWICK and KALCKAR 1943). The inhibition of the dissolved hexokinase of the diaphragm by G-6-P is dependant on the ATP concentration. Thus 8×10^{-4} M G-6-P gave 50 % inhibition at an

Table VIII.

The inhibition of hexokinase in the soluble fraction of rat diaphragm by glucose-6-phosphate at different concentrations of ATP.

Glucose-6-phosphate Molarity	With 0.0012 M ATP		With 0.0023 M ATP	
	Hexokinase activity µg glucose phosphorylated	Inhibition %	Hexokinase activity µg glucose phosphorylated	Inhibition %
0	267		368	
0.0004	172	36	320	13
0.0008	120	55	245	33
0.0016	82	69	164	55

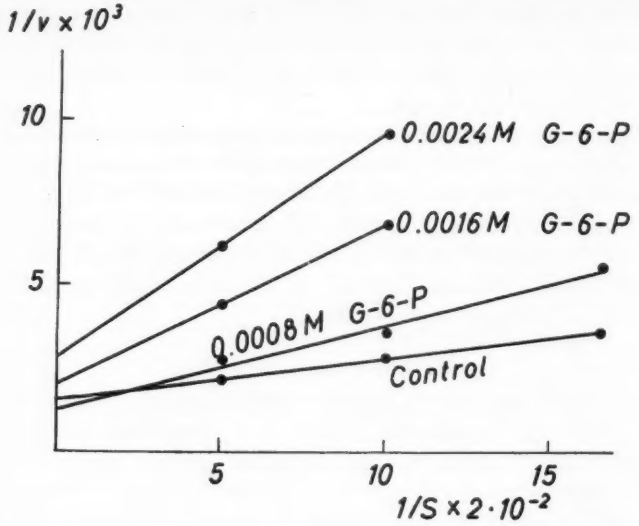

Diagram IV.
Type of inhibition of hexokinase activity by G-6-P in the soluble fraction of rat diaphragm.
v = μgm glucose phosphorylated.
s = Molar concentration of ATP.

ATP concentration of 1.2×10^{-3} M. An increase in the ATP concentration counteracted the inhibition, and at a concentration 2.3×10^{-3} M of ATP, 1.6×10^{-3} M G-6-P was necessary to produce 50 % inhibition. Additional experiments confirmed this finding and calculation according to the method of LINEWEAVER and BURK (1934) shows an inhibition of the non competitive type regarding ATP (Diagram IV). However, the soluble fraction of the diaphragm represent a crude system, and the problem requires further investigation using purified muscle hexokinase.

The Effect by 2,4-DNP.

In a series of different experiments an inhibition of the hexokinase reaction by 2,4-DNP has been observed. As shown in Diagram V the inhibition occurred in the range of concentrations 1×10^{-4} M to 5×10^{-4} M of 2,4-DNP. In the particulate fraction 50 per cent in-

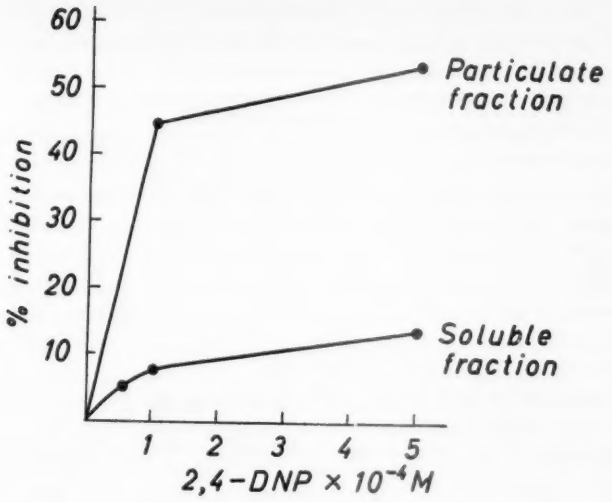

Diagram V.
The inhibitory effect by 2,4-DNP on hexokinase activity in fractions of rat diaphragm.

hibition was given by 1×10^{-4} M 2,4-DNP, while the soluble fraction was relatively resistant to 2,4-DNP. Thus, the inhibition of the hexokinase reaction in this fraction did not exceed 20 per cent even when 5×10^{-4} M of 2,4-DNP was present. These experiments were all carried out with the relatively low concentration of 1.5×10^{-3} M ATP. The influence of the ATP-concentration is shown in Table IX and X, in experiments on the particulate and soluble fractions, respectively. In the particulate fraction the inhibition by 2,4-DNP was greatly reduced when the concentration of ATP was increased from 1.5×10^{-3} M to the saturation level 6×10^{-3} M. Similar results were obtained in experiments on the soluble hexokinase. In the experiments reported, a concentration of 5×10^{-4} M 2,4-DNP was used, but essentially the same observations were made with 1×10^{-4}M 2,4-DNP. Thus, the inhibition of hexokinase reaction by 2,4-DNP to a large extent could be overcome by increasing the level of ATP.

Table IX.

The inhibition of hexokinase activity in rat diaphragm particles by 2,4-DNP at different concentrations of ATP.

ATP Molarity	Hexokinase activity		Inhibition %
	Control	5×10^{-4} M 2,4-DNP	
	μg glucose phosphorylated		
0.0015	51	26	49
0.0030	78	51	35
0.0045	86	67	22
0.0060	83	76	8

Table X.

The inhibition of the hexokinase activity in the soluble fraction of rat diaphragm by 2,4-DNP at different concentrations of ATP.

ATP Molarity	Hexokinase activity		Inhibition %
	Control	5×10^{-4} M 2,4-DNP	
	μg glucose phosphorylated		
0.0007	94	34	64
0.0014	202	166	18
0.0025	286	248	13

Discussion.

It is well known that 2,4-DNP inhibits the generation of high energy phosphate bonds associated with the transport of electrons from DPNH to the cytochrome oxidase (LOOMIS and LIPMANN 1948, CROSS, TAGGART, COVO and GREEN 1949). The explanation of this fact being that 2,4-DNP results in the breakdown of labile organic phosphorous compounds produced as initial products of the phosphorylations (TEPLY 1949, HUNTER 1951). On the other hand phosphorylations on the substrate level are usually insensitive to 2,4-DNP. One example is the coupling of phosphorylations to the oxidation of α-ketogluratate by DPN (HUNTER and SPECTOR 1948). Phosphorylations associated with the transformation of glycogen to lactic acid by the Embden-Meyerhof pathway are likewise insensitive to 2,4-DNP. The present experiments on the isolated diaphragm have thus not provided any evidence that 2,4-DNP produces accumulation of intermediary pro-

ducts in the glycolysis. Substrate from the glucose uptake and glycogen breakdown is almost converted quantitatively to lactic acid when 2,4-DNP is present.

It is therefore of interest that 2,4-DNP inhibits hexokinase reaction in the diaphragm, especially in the particulate fraction. This effect explains why 2,4-DNP inhibits the glucose uptake in the surviving diaphragm muscle. A tentative hypothesis is that 2,4-DNP produces a breakdown of energy-rich phosphate esters resulting in a reduced content of ATP in the hexokinase system. The effect is not only due to an uncoupling of oxidative phosphorylations, since 2,4-DNP under anaerobic conditions also gave a strong inhibition of the glucose uptake. 2,4-DNP probably accelerates reactions in the surviving diaphragm resulting in an increased hydrolytic cleavage of ATP, which becomes the limiting factor in the hexokinase system. The fact that 2,4-DNP accelerates the ATP ase activity in the particulate and soluble fraction of the rat diaphragm confirms this hypothesis (WALAAS, BRIGHAM and BRINCK-JOHNSEN 1956).

SOLS and CRANE (1954) observed that brain hexokinase was inhibited by ADP competitively with ATP. In our experiments this may however be of little importance. In the experiments on fractions of the diaphragm there was a relatively large excess of ATP. Further, it must be supposed that adenylate kinase will keep the ratio ATP/ADP at a constant level in the diaphragm.

The sensitivity of the cell fractions to 2,4-DNP was somewhat different. While 5×10^{-5} M 2,4-DNP inhibited the glucose uptake considerably in the surviving diaphragm, 1×10^{-4} M 2,4-DNP was required to obtain a definite inhibition of the hexokinase reaction in the particulate fraction. The soluble fraction was relatively resistant to 2,4-DNP since 5×10^{-4} M only gave slight inhibition. A similar case is that of the effect of nitrophenols on the oxidative phosphorylations and on the O_2-uptake. CLOWES and KELTCH (1951) showed that 1×10^{-5} M dinitrocresol produced uncoupling of oxidative phosphorylations in the particulate fractions of brain and liver, while phosphorylations in the water soluble fraction was almost resistant to 5×10^{-3} M dinitrocresol. PEISS and FIELD (1948) found that 2,4-DNP gave a much more marked stimulation of the O_2-consumption in brain slices than in cell free extracts of brain. Thus, the effect of 2,4-DNP on enzymatic reactions is weaker when the cell structure is destroyed.

Summary.

1. The effect of 2,4-DNP on the glucose uptake in the isolated rat diaphragm and on the hexokinase reaction in the particulate and soluble fraction of the diaphragm has been investigated.

2. 5×10^{-5} M 2,4-DNP inhibited the glucose uptake in the diaphragm both under aerobic and anaerobic conditions. 2,4-DNP blocked the stimulating effect of insulin on the glucose uptake in the diaphragm, while 2,4-DNP + adrenaline gave additive inhibition.

3. 10 % of the hexokinase was associated with particles isolated from the diaphragm, while 85 % was found in the soluble fraction. Glucose-6-phosphate inhibited the hexokinase, non competitive with ATP.

4. 1×10^{-4} M 2,4-DNP inhibited the hexokinase reaction in the particulate fraction. The soluble fraction was relatively resistant to 2,4-DNP since 5×10^{-4} M gave only slight inhibition. The inhibition of the hexokinase reaction was cancelled when the ATP-concentration in the system was increased.

5. The experiments indicate that 2,4-DNP inhibits the glucose uptake in the surviving diaphragm by stimulating the deposphorylation of ATP with subsequent reduced turn-over velocity in the hexokinase reaction.

We wish to express our thanks to Eli Lilly and Company and to the Norwegian Research Council for financial support.

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Chromatographic Separation of Histaminase and Substance P-Inactivating Enzymes in Extracts of Kidney Tissue.

By

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Since Best, for the first time in 1929, described a factor present in tissue extract which was capable of neutralizing the biologic effects of histamine, many attempts have been made to purify the enzyme termed histaminase (McHenry and Gavin 1931, Laskowski 1942, Swedin 1943, Stephenson 1943, Laskowski, Lemley and Keith 1945 and Tabor 1951). Nevertheless, no preparation pure enough to answer all the questions regarding its nature and mechanism has so far been obtained. It is still uncertain, for example, whether a whole or half a molecule oxygen is required in the catalysed oxidative deamination of the histamine molecule by the enzyme (Laskowski 1942, Swedin 1944, and Tabor 1951) and whether diamine oxidase and histaminase are identical (Zeller 1938, and Kapeller-Adler 1949).

The crude histaminase preparations contain other enzymes such as catalase and various proteolytic enzymes. It was found that histaminase preparations also inactivated bradykinin (Pereira 1952). This observation was confirmed by Hamberg and Rocha e Silva (1954),

who showed that this effect was due to a proteolytic agent, which could be distinctly separated from the histaminase activity by means of paper electrophoresis. The method used however, permitted no further studies on the degree of purity of these enzymes.

As preliminary results showed that histaminase preparations from kidney tissue according to SWEDIN (1943) also contained a factor which inactivated substance P and most likely was identical with the bradykinolytic factor, we attempted to separate the two enzymes chromatographically.

Methods.

Preparation and purification of histaminase and the proteolytic enzyme from hog kidneys. The kidneys were removed directly from the pigs in the abattoir and immediately cleaned of connective tissue and marrow, frozen to −20° C., and minced in a Turmix blender. An equal volume of saline was added and the mixture heated to 62° C for 10 minutes and filtered overnight at 4° C through ordinary filter paper. To the filtrate was added neutral acetone until the final concentration was 40 % by volume; the mixture was then centrifuged after 30 minutes. The precipitate was dissolved in the smallest possible volume of 2.5 % NaCl and centrifuged. Half a volume of neutral saturated ammonium sulphate was added to the clear filtrate, and the mixture was centrifuged after 30 minutes. The supernatant was mixed with one and a half volumes of ammonium sulphate and centrifuged after 2 hours. The precipitate was dissolved in the smallest possible volume of distilled water and dialysed in a cellophane bag against 2.5 % NaCl for 48 hours at 4° C. The NaCl solution was changed four times during the dialysis. The yield for this preparation was 8−10 %.

Determination of organic matter. Gravimetric determinations were performed where larger amounts of the enzyme solutions were available. 0.2 ml of the dialysed preparation was dried in a platinum dish, weighed and then incinerated to determine the inorganic material. Where only small amounts were available the light absorption was measured at 280 mμ of the enzyme solution diluted with the buffer which was later to be used in the biologic assay. This buffer also served as a blank. The preparations gave an absorption of $E\ \frac{1\ cm}{1\ \%} = 9$ at 280 mμ when compared with the gravimetric method.

Determination of the enzyme activity. The effect of the histaminase and proteolytic enzyme, which inactivated substance P, were determined biologically.

This was carried out on isolated guinea-pig ileum suspended in 3 ml Tyrode's solution (pH 8.2) at 38° C and aerated with pure oxygen. Standard solutions of 1 μg/ml histamine and 10 units/ml. substance P were used.

A rough determination was first made of the fractions obtained from the ion exchange column, diluted 1:10 with the elution buffer. The active fractions were then further diluted 1:100 and 1:1 000. M/150 phosphate buffer pH 6.5 was used in diluting for the histaminase assay and M/150 phosphate buffer pH 7.5 for the proteolytic assay. The reacting solutions were incubated at 37° C for one hour and the biological activity determined at once. 5—10 μg histamine phosphate per ml reacting solution was used as substrate for the histaminase determinations, and 10 units substance P per ml reacting solution for the proteolytic determinations. Substance P, which was obtained from an intestine extract by fractionation with ammonium sulphate and further purified by adsorption on aluminum hydroxide according to PERNOW (1953) had an activity of 200 units per mg dry matter.

Chromatographic separation on ion exchange columns. The ion exchange material, Dowex 50 mesh 200—300, was at first treated by washing with 2N NaOH, distilled water, 2N HCl, and again distilled water until neutral, and lastly with the buffer to be used in the adsorption of the active substance. The ion exchange material was mixed with the buffer and poured into the chromatographic tube, 0.9 cm inner diameter and 20 cm high. The volume of test solution was 0.15—0.20 ml containing 15—20 mg organic matter. This was carefully pipetted on to the surface of the column, adsorbed, washed down with 0.2 ml buffer, and covered by buffer to a height of 10 cm above the column. The upper end of the tube was connected to a reservoir containing buffer, to ensure constant flow, and the lower end closed with a capillary to regulate the speed of flow through the column. A speed of 1 drop per 90 seconds gave fractions of 0.3 ml every half hour. The temperature throughout the experiment was 20° C.

Results.

Determination of Optimum pH and Incubation Time for the Breakdown of Histamine and Substance P.

The influence of the pH on the enzymatic activity of the kidney extracts was studied over a pH range of 6—9 using M/150 phosphate buffer.

The optimum pH for the histaminase activity was found to be 6.5—6.8, and for the proteolytic factor 7.5, which is in agreement with the results of HAMBERG and ROCHA e SILVA (1954).

In order to study the incubation time necessary for optimum destruction of histamine, one of the active fractions obtained after chromatography was diluted 1:100 and incubated with histamine at 37° C for 10 to 90 minutes. The incubation was interrupted by boiling. As is indicated in fig. 1, the optimum effect was obtained after 60 minutes.

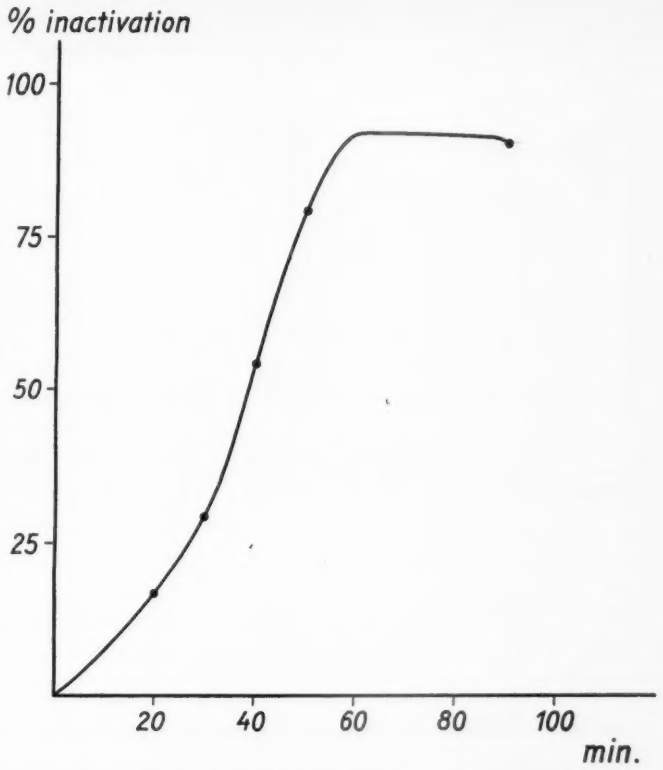

Fig. 1. Percentage destruction of histamine by a histaminase preparation purified on a Dowex 50 ion exchange column. The reacting solutions were incubated in a M/150 phosphate buffer, pH 6.5, at 37° C.

Determination on the Activity of a Purified Kidney Tissue Extract.

The organic dry material was 8.5 mg./ml., giving a light absorption at 280 mμ $E\ \frac{1\%}{1\ cm} = 9$. The extract diluted 1:1000 inactivated 1.5 μg histamine/ml./hour or 0.167 mg histamine/1 mg. organic dry matter/hour. At this dilution 500 units substance P was inactivated by 1 mg. organic dry material per hour.

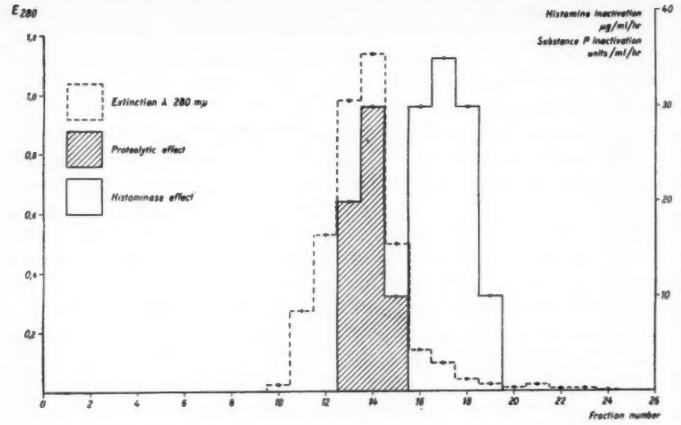

Fig. 2. Separation of the histaminase and the substance P-inactivating agent in kidney extract on an ion exchange column (Dowex 50, 0.9 × 20 cm) in a M/150 phosphate buffer, pH 7.1. Effluent volume was 0.3 ml. per 30 minutes.

Both this extract and the various fractions obtained in the separation were stored at − 20° C and retained their activity for at least one month.

Chromatographic Separation of Histaminase and Proteolytic Enzyme with Dovex 50.

M/150 phosphate buffers of different pH were used as elution solvents. At pH 6.0—6.5 there was a great loss of activity, no separation of the active components, and no purification. At pH 6.5—6.9 the yield was greater, but no separation or purification was attained. pH 7.0—7.5 gave the best results with regard to both separation and purification. At this pH range a complete separation was observed between the histaminase and the proteolytic factor. A satisfactory separation was also obtained between the histaminase and the inert protein fraction. In a typical experiment (Fig. 2) a purification of about 40 times was effected, *i.e.* 6.3 mg. histamine was inactivated by 1 mg. organic matter. The substance P-inactivating factor more or less followed the inactive protein fraction, wherefore no purification of this enzyme could be attained. The recovery of both enzymes from the ion exchange column was about 70 %.

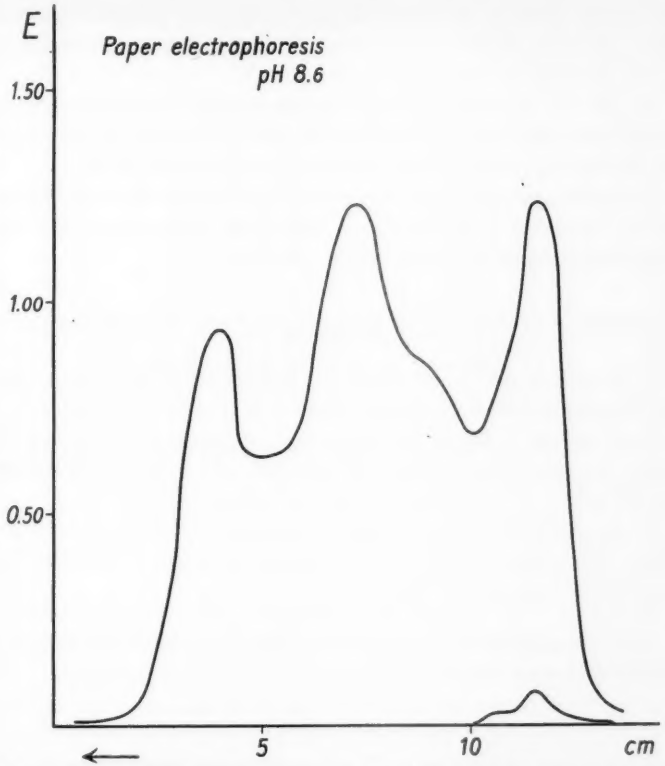

Fig. 3. Paper electrophoretic analysis of the purified kidney extract and the most active histaminase fraction obtained after chromatography, run in a barbiturate buffer, pH 8.6, at 6 volts/cm. and 2 mA. for 16 hours. The papers were developed in bromophenol blue, cut into 0.5 cm. strips and the colour eluted with a methanol/Na_2CO; mixture. The colour intensity was read in Beckman B at 595 mμ and was proportional to the protein concentration of the two preparations which had the same histamine activity.

The purest histaminase fractions obtained from the ion exchange column were compared electrophoretically with the purified kidney extract. The electrophoresis ran for 16 hours at 220 volts in a barbiturate buffer of pH 8.6, and the strips were developed in bromophenol blue. The histaminase and the inert protein fraction moved to the anode and a distinct separation of three fractions was achieved with

the kidney extract. With the chromatographically purified fractions only one spot could be observed which corresponded to the position of the histaminase activity (Fig. 3).

At pH 7.8—8.2 both the enzymes and contaminating protein were completely absorbed. All attempts at eluting the active components by altering the pH and salt concentration of the buffer failed.

Our purest preparation is therefore about 10 times purer than those so far described in the literature and 1.000 times purer than the commercial preparation, "Torantil" (BAYER).

Summary.

1. A method for the extraction and purification of histaminase and a substance P-inactivating factor from hog kidneys is described.

2. A complete separation of the two enzymes was achieved by using ion exchange columns (Dowex 50) and a M/150 phosphate buffer (pH 7.1).

3. By this method a satisfactory separation was also attained between the histaminase and inactive protein, giving a further purification of the enzyme of 40 times.

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Contribution of the Sarcolemma to the Force Exerted by Resting Muscle of Insects.

By

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The resistance of resting muscle to stretch depends on the resistance of the contractile substance and of parallel and series elastic components. According to RAMSEY and STREET (1940) and RAMSEY (1947), the load-extension diagram of the resting muscle is solely determined by the sarcolemma and the interior of the fibre is assumed to act as a sol which is gelled by contraction. CASELLA's experiments (1951), on the other hand, indicated that the sarcolemma of the isolated frog's muscle fibre does not exert an appreciable influence on the resting force before the muscle is stretched to about 150 per cent of its equilibrium length. In total vertebrate muscle, conditions are still more complex since parallel elastic components other than the sarcolemma exert tension. HILL (1949) recommends, therefore, working only with muscle at a length shorter than the resting length in the body in order to ensure slackness of these components.

In interpreting mechanical changes in the contracting muscle, it is essential to know the influence of passive elastic components. It is, therefore, of interest to study the load-extension relationship in resting flight muscles of insects because of their simple arrangements: the fibres run parallel from origin to insertion; there is practically no

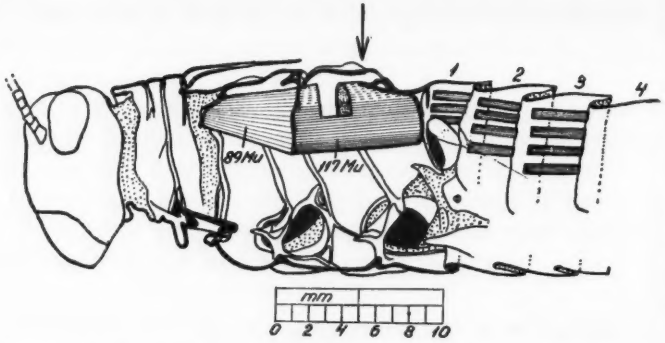

Fig. 1. Sagittal section of thorax and anterior part of abdomen of *Schistocerca gregaria*, showing the muscles used.

117 Mu (MISRA 1950) is the dorsal longitudinal flight muscle of the metathorax (no. 112 in SNODGRASS 1929). The part which was not used is indicated by broken hatching. 89 Mu is the corresponding muscle in the mesothorax. The abdominal muscles are suspended between the third (3) and the fourth (4) abdominal terga. The arrow shows where the section in figure 4 was cut.

connective or tendinous tissue, so that parallel and series elastic components are virtually absent outside the fibres themselves.

The load-extension relationship was studied in total flight muscles of locusts, in their isolated fibres and in empty sarcolemma tubes of slightly damaged fibres. The results are compared with measurements of the dorsal longitudinal muscles in the abdomen and of the fibrillar flight muscle of *Hydrophilus*.

Material and Methods.

Material. Adult female desert locusts (*Schistocerca gregaria* Forskål, *phase gregaria*) were used in the experiments. The insects were cage-bred at the Anti-Locust Research Centre, London; a number of anatomical and physiological data have been published (WEIS-FOGH 1952). The flight muscle used is the dorsal longitudinal (indirect) depressor of the hind wing (117 Mu in fig. 1). It is suspended between the second and third phragma which both originate from the elastic metanotum. Like other flight muscles in locusts, the alterations in length during passive stretch or active contraction rarely exceed five per cent of the *length in the body* L_b, *i.e.* of the length in the longitudinally cut thorax when the wings remain folded. The abdominal muscles used are the homologous dorsal longitudinal muscles, suspended between the third and fourth abdominal terga. Since

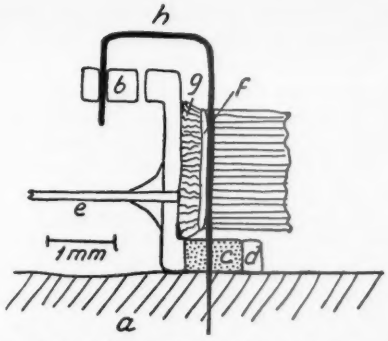

Fig. 2. Suspension of the flight muscle (117 Mu). (a) bottom of preparation dish; (b) upper platform with drilled holes; (c) cork slab of lower platform, fixed between the middle piece and the frame (d); (e) stainless steel rod to myograph. Between the middle piece and the cuticular phragma (f) are squeezed the remains of the mesothoracic flight muscle 89 Mu (g); (h) stainless steel hook keeping the muscle in place.

the length of these muscles changes considerably under respiratory needs, pregnancy etc., they have no constant resting length in the body. In some experiments bundles of fibres were isolated from the dorsal longitudinal wing muscle of the water beetle *Hydrophilus piceus* L. (*Hydrous p.*).

Preparation. The muscle was removed from the insect together with the adjacent cuticular structures and placed in a saline solution (10° C) containing 175 mM Na^+, 5 mM K^+, 2 mM Ca^{++}, 161 mM Cl^-, 10 mM PO_4^-, 8 mM HCO_3^- (cf. BUCHTHAL, WEIS-FOGH, & ROSENFALCK). After saturation of the solution with 95 per cent O_2 and 5 per cent CO_2, its pH was adjusted to between 6.8 and 6.9. When total muscles were used, bubbling with CO_2 and O_2 was continued throughout the experiments. This was not considered necessary with isolated fibres, but the saline solution was then renewed several times during the experiments.

The *flight muscle* was isolated from all structures, except for the phragmata and the postnotum, care being taken to avoid injury to the tracheal supply. The muscle was suspended and slightly stretched between two small Z-shaped clamps of plastic (fig. 2) fixed to the floor of the preparation dish *a*. Each clamp has an upper platform *b* with numerous holes in it, 0.2 *mm* in diameter, and a lower platform, consisting of a cork slab *c* surrounded by a frame *d*. The vertical middle piece connecting the two is attached to the myograph by means of a horizontal stainless steel rod *e* of 0.2 *mm* diameter. The phragma *f* was pressed against the vertical section by leading the pointed branch of a thin stainless steel hook *h* (0.1 *mm* diameter) along the cuticle and well through the cork slab; at the same time,

the upper bent part of the hook was made to slip into a hole in the upper platform. In this way each phragma was clamped firmly at three (second phragma) or two (third phragma) places against the vertical section of the suspending clamps, preventing slack. The dorsal part of the muscle (marked by broken lines in fig. 1) was now removed, leaving a bundle of parallel fibres of practically the same length and firmly suspended at both ends.

The *abdominal muscle* consists of a number of parallel fibre bundles situated below the body wall and separated from it by small flat cushions of adipose tissue (fig. 6). The two tergites to which it is attached were reduced in size; the muscle was suspended and slightly stretched in the preparation dish. After careful removal of that part of the cuticle which is situated between the attachments, the terga were fixed to two clamps by means of small cork strips and thin needles.

Three to five fibres were isolated from the flight muscle and single fibres from the abdominal muscle. The preparation and suspension proceeded as with the whole muscles. In isolating small bundles of fibres or a single fibre, great care is necessary to avoid pulling the small tracheae and thereby damage the preparation. The excitability of the sample was checked repeatedly. In the flight muscle the tracheal supply was so dense that isolation of undamaged single fibres proved unsuccessful and bundles consisting of a few fibres were used instead.

Empty sarcolemma tubes were obtained by squeezing the slightly stretched fibre gently between two tapering glass rods. After some minutes, the stretched, damaged fibrillar content of the fibre broke and retracted at each end to a shorter length, leaving a distinct sarcolemma tube in between. The length of the empty section generally amounted to about one fourth of the length of the slightly stretched fibre. Electrical stimuli could still produce local contractions in the undamaged portions of the abdominal fibre. In the flight muscle, the outer diameter of the sarcolemma tube was the same as the original diameter of the intact fibre. In fibres from the abdominal muscles, the empty sarcolemma tube constricted like that of frog muscle (CASELLA 1951).

Apparatus. The resting force of *isolated fibres* was measured by means of a calibrated twin cantilever consisting of two thin steel blades, 10 *cm* in length. A small dash-pot with glycerol provided damping. The deflections of the cantilever were determined microscopically with an eyepiece micrometer, and the fibre length was measured with a measuring microscope.

The force produced by stretching the *total muscle* was recorded by means of a condenser myograph (BUCHTHAL 1942; BUCHTHAL, KAISER & KNAPPEIS 1944); the movable plate was attached to the horizontal steel rod of the one muscle clamp. The change in capacity caused by the stretching force was transformed to a change in voltage by means of a high frequency circuit, linearity between force and voltage being ascertained within the range of operation. The other muscle clamp was fixed to a travelling sledge, moved by a micrometer screw to introduce varying degrees of stretch. The initial length was estimated with an accuracy of $\pm$ 0.05 *mm*. The force produced by different degrees of stretch was determined with an accuracy

of 5 per cent of the maximum force. The isometric force of a twitch was checked before and during the experiments to control the state of the muscle.

Procedure. The length of the *flight muscle* was measured *in situ*, and the isolated muscle was stretched to this length for at least ten minutes. Then the muscle was loaded by about 0.5 *g* and the new length was referred to as the equilibrium length L_o, *i.e.* the length corresponding to a stretching force of about 2 per cent of the yielding force (Table II). The term yielding force indicates the force which just causes an irreversible increase in fibre length. After each alteration in length, time was allowed for adjustment to constant stress (2 to 5 minutes). The equilibrium length of the *abdominal muscle* was defined as the length attained at five per cent of the yielding load. This also applies to *isolated fibres* of both types of muscle. The structural state of the fibres was observed under the microscope throughout the experiment. The experiments on total muscle were performed at 5° to 10° C; those on isolated fibres at 18° to 20° C.

Histological sections. The muscles were fixed *in situ* for 24 hours in four per cent neutral formaldehyde saline, washed in tap water for another 24 hours, soaked in increasing concentrations of gelatine solution at 37° C for several days and embedded in 25 per cent gelatine. Thereafter, the gelatine-muscle blocks were fixed in 10 per cent formaldehyde, and 15 μ frozen sections were cut and mounted in glycerol jelly between coverslips. The shrinking produced by this method was negligible.

Morphology.

Female locusts of nearly the same size were selected for the experiments. Since cage-bred locusts are extraordinarily similar in geometry (Weis-Fogh 1952), the muscle preparations were of uniform dimensions, as indicated in Table I. After removal of the dorsal part of the *flight muscle* (broken hatching in fig. 1), the length of the fibres in any one preparation varies about ten per cent. At "equilibrium length" the long fibres are therefore slack. The fibres run without interruption and without any significant change in diameter from one phragma to the other. They do not taper at the ends and the cross striation continues straight to the attachments without intercalated tendons (fig. 3). Figure 4 shows a cross section of the total muscle at the level of the vertical arrow in figure 1. In insects of standard size, the cross sectional area of the whole muscle of one side is $2.68 \cdot 10^{-3}$ cm^2; it contains about 2300 fibres. The figures in Table I show the values after removal of the shorter dorsal fibres. The cross section of the fibres is polygonal and of fairly uniform area (1200 μ^2). The fibres are packed in bundles held together by tracheae; these bundles are delimited by larger tracheae and blood-filled passages, but there is practically no connective tissue. About eighty per cent of the volume of the muscle consists of fibres, the remainder being occupied by tracheae or blood.

The preparation of the *abdominal muscle* consists of four or five parallel

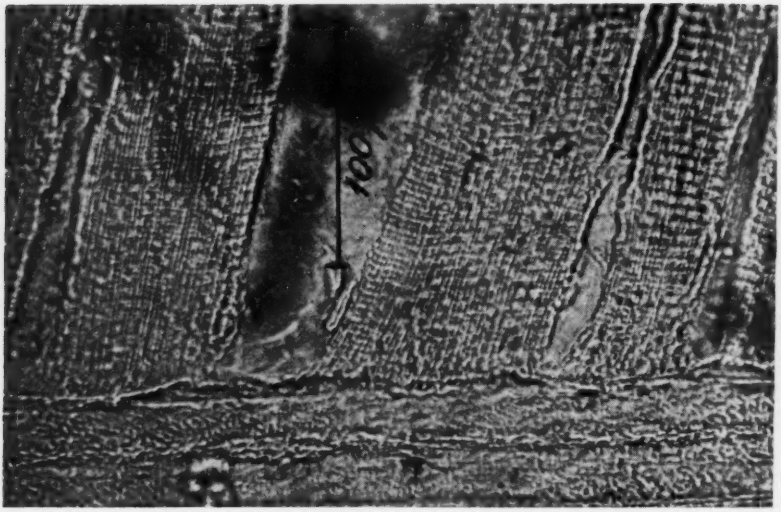

Fig. 3. The attachment of the flight muscle fibres at the second phragma. The cross striation continues right up to the cuticle; the fibres do not taper.

bundles of fibres (indicated in fig. 1) so that within the body, the width is nearly the same as the length. The equilibrium length varies by about 15 to 20 per cent from the shortest to the longest bundle in a preparation. As in the flight muscle, the fibres are directly attached to the cuticle without tendons (fig. 5) but their shape and cross sectional area vary considerably (fig. 6). Each bundle consists of 20 to 25 fibres. The average fibre has approximately the same cross sectional area in both types of muscle (1200 μ^2); it is considerably smaller than the average frog muscle fibre (11 000 μ^2; CASELLA 1951).

Conclusion. The morphology of the two locust muscles favours an investigation of their mechanical properties because: (1) the fibres extend from the one cuticular attachment to the other; (2) there is no tendon or other serially arranged connective tissue, the cross striation continuing throughout; (3) the fibres do not taper (fig. 3) or the tapering is but small (fig. 5); (4) there is no connective tissue in parallel with the fibres apart from tracheae and parts of the fat body which have very little longitudinal stiffness compared with the fibres. The mechanical resistance to stretch can therefore be attributed solely to the combined effect of the sarcolemma plus the content of the fibres.

Table I.

Dimensions of the muscles (Schistocerca gregaria)

The number of preparations used is given in brackets.

	Flight muscle (dorsal longitudinal)	Abdominal muscle (dorsal longitudinal)
Length (*cm*):		
equilibrium length L_o	0.65 ± 0.01 (9)	0.33 ± 0.01 (9)
length in the body L_b	0.76 ± 0.01 (9)	ca. 0.43
Total cross sectional area of fibres at length in the body (cm^2)	$1.85 \cdot 10^{-2}$ (3)	$1.3 \cdot 10^{-2}$ (8)
Number of fibres per preparation	1540 (1)	110 (8)
Average cross sectional area of a fibre at length in the body (μ^2)	1200	1180
Average fibre diameter (circular cross section) at length in the body (μ)	39 [1]	37 [2]

[1] Small variation, polygonal cross section.
[2] Large variation, often tape-shaped fibres.

When whole muscle is used instead of small fibre bundles, the different lengths of the fibres must be taken into account. They can easily be estimated, however.

Results.

Resting flight muscles of insects differ markedly from other muscles in that they are much less extensible. Table II shows the yielding length and the ultimate length of various muscles. In experiments with

Table II.

Extensibility of resting muscles.

	Flight muscle		Abdominal muscle of *Schistocerca*	Frog muscle fibres
	Schistocerca	*Hydrophilus* [2]		
'Yielding' length:				
in per cent of L_o	160	120	210	220
in per cent of L_b	140	110	160	—
Greatest observed length:				
in per cent of L_o	190 [1]	135	160	320

[1] Most fibres broken.
[2] See page 358.

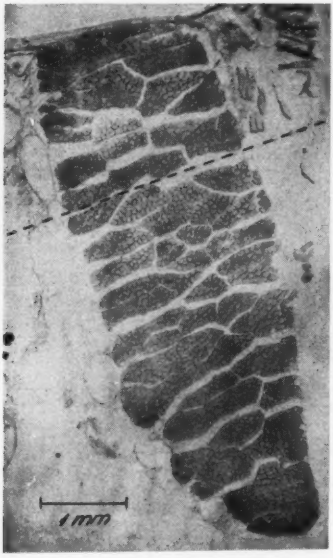

Fig. 4. Transverse section of the flight muscle at the level of the arrow in figure 1. The postnotum is seen above and the free ventral edge of the muscles below. Note that the fibres are grouped in bundles held together by thin tracheae but practically without any connective tissue in between. The part of the muscle below the broken line is used in the experiments.

isolated fibres, care was taken to avoid irreversible deformations, *i.e.* the flight muscle fibres were not stretched to beyond 150 per cent and the abdominal fibres not to beyond 190 per cent of equilibrium length.

1. Isolated Fibres.

Flight muscle of Schistocerca. A typical experiment with three undamaged fibres is illustrated by the full curve in figure 7. It shows that the load as a function of extension followed a steep and nearly rectilinear course and that the equilibrium length remained unaltered within the applied range of stretch. The difference in force with increasing and decreasing length (arrows) was avoided in later experiments by allowing sufficient time for adjustment, viz. 2 to 3 minutes. The lower curve in figure 8 is the average load-extension relationship found in eight preparations, each containing 3 to 5 un-

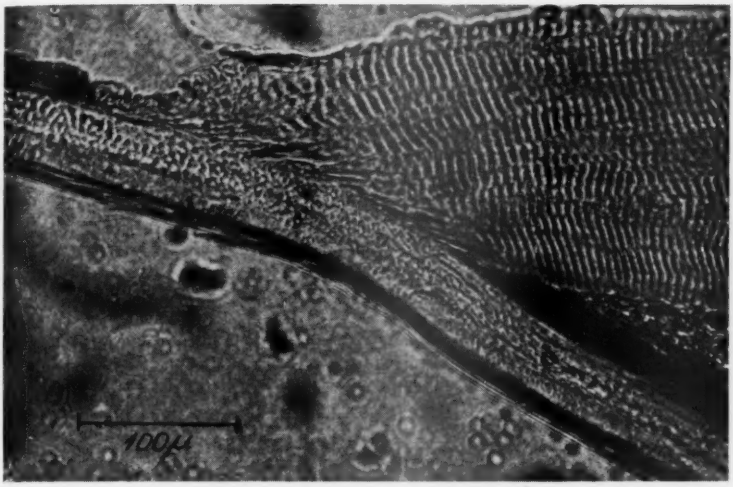

Fig. 5. The attachment of an abdominal muscle fibre to the tergal plate. The cross striation extends to within a few microns from the cuticle and there is only very little tapering.

damaged fibres with an average diameter of 50 μ. The results are calculated as the force exerted per fibre and the standard errors are indicated by vertical bars. The curve has the same steep slope as that in figure 7; from 110 per cent to 150 per cent of the equilibrium length it did not deviate significantly from a straight line, indicating that the resting force is proportional to the stretch. In these experiments, the yielding force per fibre was above 40 *mg*, probably between 50 and 60 *mg*.

A number of fibre bundles were slightly stretched and empty sarcolemma tubes were prepared. Contrary to frog fibres (CASELLA 1951), the equilibrium length thereby decreased from 6.0 *mm* to 5.5 *mm* but the diameter of the empty tube remained the same as before the injury (50 μ). On the other hand, the diameter of the cross striated content increased to 76 μ and that of the granulated, "amorphous" parts at the transition between the two to 92 μ. The treatment apparently caused the fibres to swell to 1.5 or 2 times their original volume, leaving the initial diameter of the sarcolemma tube the same as before the injury. When stretched to breaking, the break always occurred

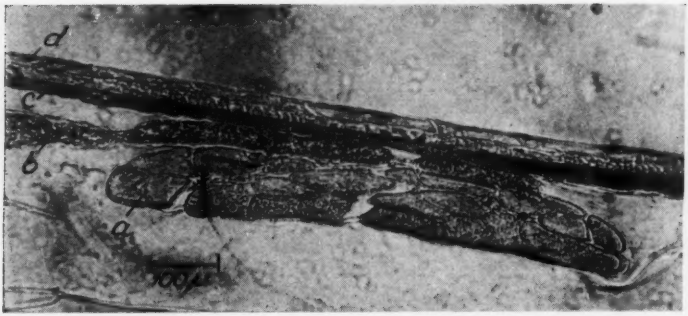

Fig. 6. Cross section of a bundle of abdominal muscle fibres (a) lying beneath the hypoderm (c) of the tergum (d) and protected by the adipose tissue (b). The size of the fibres differs considerably.

somewhere in the empty sarcolemma tube. The upper curve in figure 8 shows the load-extension relationship of ten preparations, the force being expressed in *mg* per fibre. The lengths of the bars indicate the scattering (standard error) of maximum force and length of the injured fibres. The most interesting result is the relatively small breaking force of the sarcolemma, 12.3 ± 1.2 *mg* (SD = 3.7 *mg*); it is only one fourth to one third of the yielding force of the intact fibre. One might object that the sarcolemma has been severely injured by the preparation and no direct proof against this statement can be forwarded. However, severe and uncontrollable damage of the sarcolemma could hardly fail to cause great variations in the breaking force, which is in contrast to the experimental findings. For this reason it was concluded that the sarcolemma could not be responsible for more than about one third or one fourth of the *maximum* resting force of a flight muscle fibre.

It must be emphasized that the load-extension relationship of these preparations (upper curve in figure 8) does not show the properties of the sarcolemma as such but of the whole fibre, the injured parts as well as the uninjured. Up to about 120 per cent of the equilibrium length, the course was rectilinear but "yielding" then occurred, as is illustrated by the broken line in figure 7. One can therefore only make a rough estimate of how much the sarcolemma contributes to the resting force at ordinary degrees of stretch. The equilibrium length of the injured fibre averaged 5.5 *mm*; 1.4 *mm* corresponded to the

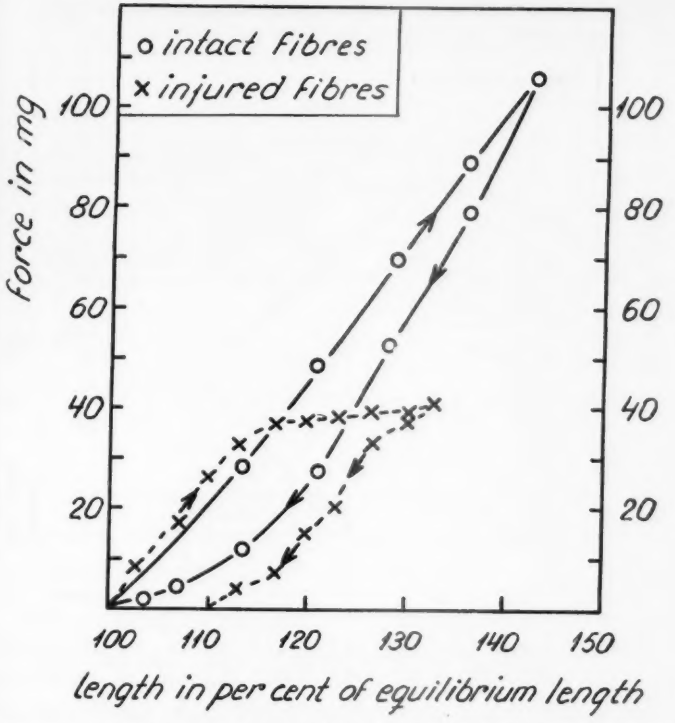

Fig. 7. Stretch and release of *three* uninjured flight muscle fibres (full line) and of *four* other fibres with empty sarcolemma tubes (broken line).

empty tube and about 4 *mm* was striated and appeared to be undamaged. The length in the body, L_b, is 115 to 120 per cent of the equilibrium length of the uninjured fibre; the force exerted by the sarcolemma alone can be estimated if a known part of the tube is given this relative elongation. We may now consider three extreme possibilities. (a) The sarcolemma is completely disentangled from the content so that it is subjected to the same longitudinal tension at all cross sections. But, in the experiments, the sarcolemma could only be stretched to 7.8 *mm* before it broke, while the breaking length of the uninjured fibres was 9.5 *mm*. It is therefore hardly free of the striated interior. (b) The sarcolemma is fixed to the undamaged parts

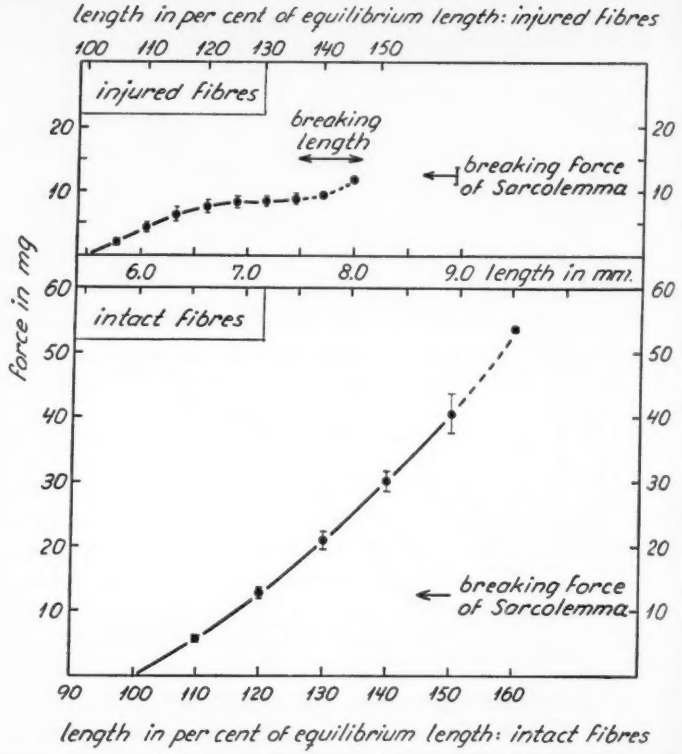

Fig. 8. Load-extension diagram of uninjured (lower curve) and injured (upper curve) flight muscle fibres. Each preparation consisted of three to five fibres. The markings show the average force per fibre, the standard error being indicated. The arrows show the average breaking force of one sarcolemma tube and the breaking length of the preparation (standard error indicated).

Abscissa: length of fibres in *mm.* (middle scale) and in per cent of the equilibrium length of injured and intact fibres (upper and lower scales respectively).
ordinate: *mg* load per fibre.

of the content and these parts are considered very stiff as compared with the empty tube. In that case the initial part of the load-extension relationship indicates that the share of the sarcolemma amounts to about 20 per cent of the resting force measured at length L_b. Finally, (c) if the sarcolemma is fixed to the undamaged parts and these parts have the same stiffness as the intact fibre, its contribution will be 30 per cent at length L_b.

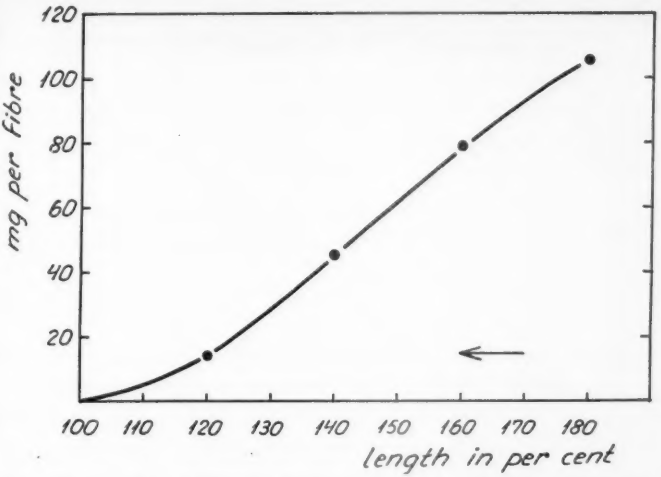

Fig. 9. The average load-extension diagram of uninjured single abdominal muscle fibres of varying cross-sectional area. The average breaking force of the empty sarcolemma tubes from the *same* fibres is shown by the horizontal arrow.

There are therefore two pieces of evidence in favour of estimating the contribution of the sarcolemma to about one third of the resting force of the normal fibre, namely the breaking force of the tube and the initial course of the load-extension diagram of the injured fibre (upper curve in figure 8).

Abdominal muscle of Schistocerca. The fibres varied considerably in diameter and thus in force. The average load-extension relationship of 13 large, intact single fibres is plotted in figure 9. Again, the resting force varied nearly linearly with the stretch at lengths exceeding 120 per cent of the equilibrium length. The average yielding force was larger than 110 *mg*, (120 to 130 *mg*, length 205 per cent). However, the ultimate force of the empty sarcolemma tubes of the *same* muscle fibres only averaged 15 ± 3 *mg* (SD = 8 *mg*). At the highest degrees of stretch, therefore, the sarcolemma could not contribute by more than one eighth of the resting force.

In some cases, a normally looking, untreated fibre gave normal resistance to stretch until its content suddenly broke, leaving an empty sarcolemma tube between its two ends. The force immediately decreased towards zero; upon subsequent stretching the preparation

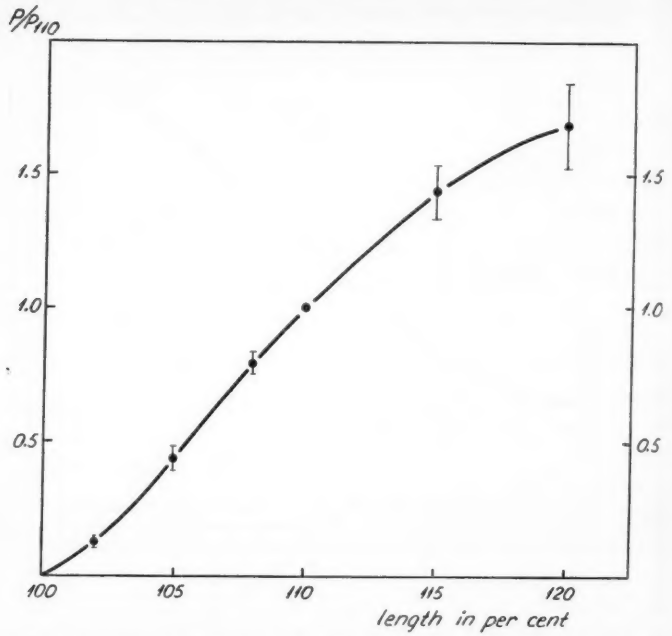

Fig. 10. Load-extension diagram of small fibre bundles isolated from the indirect depressor of the hindwings of *Hydrophilus piceus*. Time of adjustment at each length 2 *min*, 28° C. The vertical bars indicate the standard error of the mean. *Ordinate:* load in relative units, with load at length 110 per cent $L_o = 1.0$. *Abscissa:* length in per cent of equilibrium length L_o.

thereafter followed the usual load-extension relationship of fibres with empty sarcolemma tubes as produced by squeezing the content. Such observations as well as the low breaking force of the sarcolemma show that the main resistance to stretch is due to the striated content of the abdominal muscle fibres. The sarcolemma can hardly contribute by more than 10 to 15 per cent of the total force.

Flight muscle of Hydrophilus. The indirect depressor of the hindwings of the water-beetle *Hydrophilus piceus* (*Hydrous p.*) is of a transitional type between an ordinary striated muscle and the so called fibrillar flight muscles in higher insect orders (cf. TIEGS 1955). The big fibrils are assembled into 'fibres' by what appear to be the meshy remains of a sarcolemma. In any case, we were unable to find

Table III.

Average breaking force of the sarcolemma.

Compared with the 'yielding' force of undamaged locust fibres.

	Flight muscle	Abdominal muscle
Breaking force of sarcolemma (*mg*).....	12.3 ± 1.2	15 ± 3
Average 'yielding' force of intact fibre (*mg*)....	> 40	> 110

any sheet which was comparable in strength with the tough membrane surrounding the fibres of the other muscles. Recent studies with the electron microscope have confirmed this (EDWARDS, SANTOS, SANTOS and SAWAYA 1954). Provided the sarcolemma is the main structural component which offers resistance to stretch (RAMSEY and STREET 1940), we should expect to find a very soft muscle. On the contrary, the average load-extension curve of seven preparations of isolated bundles of 'fibres' distinctly shows that this muscle exerts an appreciable resistance to stretch (fig. 10). At 28° C, the average breaking force per 'fibre' (100 μ in diameter) was 105 ± 22 *mg*; the breaking length was only 134 ± 3 per cent of the equilibrium length. Yielding started at a length of 115 to 120 per cent. In this highly specialized muscle, neither the resistance to stretch nor the small extensibility could be attributed to the sarcolemma.

Conclusion. In Hymenoptera and Diptera, the fibrillar flight muscles have only remains of a sarcolemma, if any at all; yet they offer considerable resistance to stretch. These elastic forces must be ascribed to the fibrils. The three muscles investigated here represent a sequence of decreasing relative strength of the sarcolemma. In locust muscles, its role is summarized in Table III. The fibrillar content must therefore contain elastic elements, also in the non-active state.

2. Whole Muscles of Locusts.

Figures 11 and 12 show the average load-extension relationship of flight muscle and abdominal muscle respectively. In the flight muscle, the length in the body L_b corresponded to 117 per cent of the equilibrium length L_o, *i.e.* to 0.76 ± 0.01 *mm* (see Table I) and a load of 4 to 6 *g*. From 117 per cent of L_o and until yielding occurred at 160

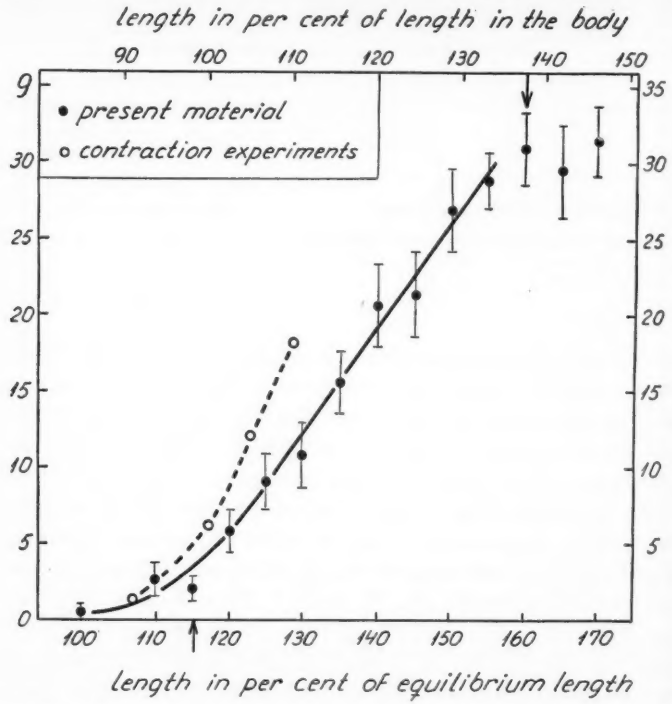
length in per cent of length in the body
90 100 110 120 130 140 150
• present moterial
o contraction experiments
0 5 10 15 20 25 30 35
100 110 120 130 140 150 160 170
length in per cent of equilibrium length

Fig. 11. Full line: The load-extension diagram of 9 resting flight muscles. The vertical lines indicate the standard error of the mean. Between the arrows, *i.e.* from the length in the body (4 *g* of load) and until "yielding' begins at 30 *g*, the relationship is nearly linear. Broken line: Similar curve from 9 muscles used in contraction experiments (BUCHTHAL, WEIS-FOGH and ROSENFALCK).

per cent of L_o, the load-extension relationship was rectilinear. This was also the case in the single curves, from which the averages were derived; it also corresponds to the experiments with isolated fibres. The absence of linearity at low degrees of stretch may be caused by the different initial lengths of the fibres, some being slack. The broken curve is derived from muscles used in contraction experiments where the method of suspension tended to damage fewer fibres than the present type (BUCHTHAL, WEIS-FOGH and ROSENFALCK). Since the average size of the muscles was identical in the two groups, the results indicate that the broken curve represents muscles in which

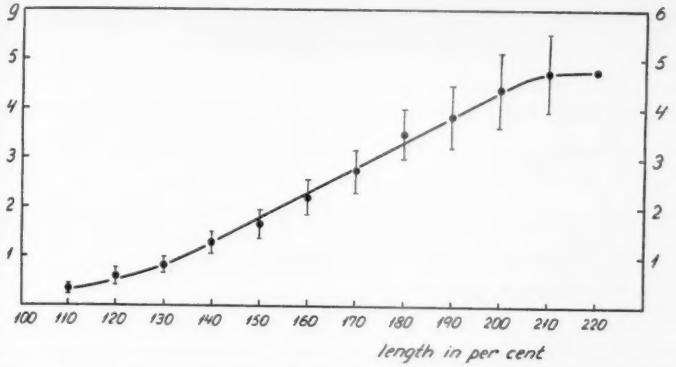

Fig. 12. The load-extension diagram of abdominal muscles when recalculated to the average area of the preparations. The vertical lines indicate the standard errors of the mean. As in the flight muscles the relationship is nearly linear between the 'yielding' load of 4.8 *g* and down to 15 per cent of that load.

30 per cent more fibres were left undamaged. The abdominal muscle (fig. 12) could be stretched to 200 to 210 per cent of its equilibrium length before yielding took place; above 130 per cent the rectilinear course was as pronounced as in the flight muscles.

The various muscles are compared in Table IV. The maximum resting tension was calculated partly referred to the cross sectional

Table IV.

Resting force and tension of locust muscles at 'yielding' length.

Compared with the maximum values observed in frog muscle fibres (CASELLA 1951). The figures in brackets are extrapolated from the broken curve in figure 11. The figures in the column "content" are obta ned by subtraction of the maximum force exerted by the sarcolemma and are but rough estimates.

	Flight muscle		Abdominal muscle		Frog muscle	
	Total	Content	To-tal	Con-tent	To-tal	Con-tent
Maximum force (*g*)	30 (49)	20 (32)	4.8	4.2	—	—
Maximum tension ($kg \cdot cm^{-2}$):						
referred to total cross sectional area at equilibrium length	1.4 (2.3)	0.9 (1.5)	2.8	2.4	5.5	1.9
referred to cross sectional area at yielding or maximum length	2.3 (3.7)	1.5 (2.4)	6.0	5.1	16.5	5.7

Table V.

Static longitudinal modulus of elasticity.

Flight and abdominal muscles of *Schistocerca gregaria.*

Muscle	Elasticity modulus ($kg \cdot cm^{-2}$) at different lengths				
	Length:				
	100 %	110 %	120 %	130 %	140 % of L_b
Dorsal longitudinal flight muscle..	7.3	7.6	7.8	8.1	8.3
Dorsal longitudinal abdominal muscle.....	5.2	6.4	7.6	8.9	10.4

area at equilibrium length and partly as the true maximum tension of the fibres at the yielding point. The sarcolemma having an insignificant cross sectional area compared with the content, the tension of the fibrils could be roughly estimated by subtracting the maximum force exerted by the sarcolemma. It is seen that, as to ultimate strength, there is hardly any significant difference between flight muscle and frog muscle. The content of the abdominal fibres could stand somewhat higher tensions (max. 5 $kg \cdot cm^{-2}$). The static longitudinal modulus of elasticity varied with the degree of stretch (Table V), but was of the same order of magnitude as in frog's muscle (BUCHTHAL, KAISER and ROSENFALCK 1951). However, the variation with stretch was much smaller in insect than in amphibian muscles (BUCHTHAL 1942) and it is tempting to ascribe this difference to the effect of the stronger sarcolemma of frog muscle fibres, rather than to any fundamental difference in the contractile substance.

Conclusions and Summary.

1. Many insect muscles are composed of parallel, non-tapering fibres with little connective or tendinous tissue to influence the forces measured during rest and activity. This applies to the three muscles used here: (a) the dorsal longitudinal depressor of the hindwings of the desert locust (*Schistocerca gregaria* Forskål); (b) the dorsal longitudinal abdominal muscle of the same insect; and (c) the dorsal longitudinal wing depressor of the water beetle *Hydrophilus piceus* L.

(= *Hydrous p.*). The relevant morphological data are given in Table I and figures 3 to 6.

2. Both types of flight muscles are much less extensible (passive stretch) than other striated muscles but, in locusts, the static modulus of longitudinal elasticity is of the same order of magnitude (about 8 $kg \cdot cm^{-2}$) as in the abdominal muscle and in frog muscle.

3. The sarcolemma is present as a tough membrane in locust muscle; slight squeezing of the stretched fibres produces empty sarcolemma tubes. In the flight muscle of *Hydrophilus* the sarcolemma, if present, has no mechanical strength compared with that of the fibrils.

4. In contrast to frog muscle, the breaking force of the empty sarcolemma tube is several times less than that of the intact locust fibre. In spite of its reduced sarcolemma, *Hydrophilus* flight muscle exerts a considerable resistance to passive stretch. In insect muscle, therefore, the content of the fibres is responsible for the major part of the resistance to passive stretch.

5. The resistance to stretch must either be due to passive-elastic elements *inside* the fibre and parallel to the non-elastic contractile elements, or the contractile system must be elastic even in the non-active state.

Acknowledgements. Thanks are due to Miss BODIL ASMUSSEN for her able assistance and to Dr. D. STEGWEE, Amsterdam, for providing us with living *Hydrophilus*. The experiments were supported by the *Anti-Locust Research Centre*, London, by the *Danish State Research Foundation*, and by the *Michaelsen Foundation*.

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The Content of Mast Cells in the Pleural Membranes, Pericardium and Liver Capsule of Cattle and Horse.

A comparison with earlier observations and assays of heparin and of histamine in these tissues.

By

Otto Wegelius

Received 7 October 1955.

Obviously the heparin originates in Ehrlich's mast cells in the connective tissue. Thanks to Jorpes' observation that heparin gives a metachromasia with toluidine blue, HOLMGREN was able to localize this metachromasia in histological sections to the mast cells. In subsequent work it was proved by HOLMGREN and WILANDER (1937) and by JORPES, HOLMGREN and WILANDER (1937) that the heparin content of different tissues to a large extent coincided with the number of mast cells present in them. Such examinations were conducted, inter alia, on the blood vessels.

During these studies HOLMGREN and WILANDER (1937) found that the liver capsule of cattle showed an unusually high content of mast cells, much higher than the parenchyma of the liver. The number of mast cells in the same tissues of the horse was not of the same order as the corresponding number in cattle. The amount of heparin present in these tissues was assayed by WILANDER (1938) and the results showed complete coincidence with the content of mast cells. The liver capsule of cattle showed the highest content of heparin and its quantity was ten times that of the liver parenchyma.

During the last years RILEY and WEST (1952) presented evidence of

a striking positive correlation between the mast cell content of some normal tissues and the amount of histamine that can be extracted from them. Histamine liberators showed an affinity to mast cells, which split up under their influence (RILEY 1953). When cells were disrupted, a higher percentage of histamine was observed (RILEY 1953, FAWCETT 1954, BENDITT, BADER, ARASE, COLEY and LAM 1954). A similar effect on the mast cells has later been found to be exerted by immune serum, applied locally (WEGELIUS, HJELMMAN and WASASTJERNA 1955). To a certain degree there was a clear parallelism between the heparin and histamine content in the normal tissues of the dog. The same was the case in the mastocytomata of the dog (CASS, RILEY and WEST 1954).

In their further studies RILEY and WEST (1953) came to the conclusion that mast cells and histamine are concentrated in the liver capsule of the ox, sheep and cow and in the pleura of the ox, pig and dog. They made the statement that enormous numbers of mast cells packed with metachromatic granules are easily demonstratable in fresh spreads of ox pleura. The histamine content ranged from 200 to 280 μg/g.

These statements give the impression that the ox pericardium and the pleural membranes of cattle are as good sources of heparin as the ox liver capsule. This is, however, not the case. Because of these statements MAGNUSSON, working in Jorpes' laboratory in Stockholm, tried to isolate heparin from the pleura of the ox, but failed (personal communication to the author). No ester sulphates were present in the pleural membranes and consequently no heparin could be expected. At the request of professor Jorpes I undertook to count the number of mast cells in these tissues.

Materials and Methods.

The material (Table I) consisted of 11 cows and 11 horses. The age of the animals was ascertained through observations of the changes in their jaws. The samples were taken from newly slaughtered animals and were immediately placed in fixative (4 % basic lead acetate).

Samples were taken from all the animals from:

pleura parietalis, on the left, from the lateral part of the thorax wall

pleura pulmonalis, from the corresponding place on the surface of the lung

pericardium, on the left

capsula hepatis from the visceral surface of the liver, on the left.

Table I.

Cattle			Horse		
No.	Age years	Weight kg	No.	Age years	Weight kg
1	2	215	1	1.5	125
2	3	270	2	2	135
3	4	240	3	7	325
4	7	170	4	12	300
5	7	220	5	12	245
6	7	175	6	14	275
7	7	190	7	14	320
8	7	335	8	15	325
9	12	160	9	16	270
10	12	140	10	17	290
11	12	220	11	20	265

The samples were cut vertically to the surface, with a thickness of 5 μ, and were stained in 1 % toluidine blue solution. The mast cells were counted per mm^2, and only cells with intact nuclei or nuclear segment were counted. The shrinking percentage in basic lead acetate (20—25 %) found by HJELMMAN (1952) in different tissues, is not considered in the tables.

Using FLODERUS' formula

$$X = n\frac{1000}{a + d - 2h}$$

the number of mast cells per mm^2 was recalculated to express the number of cells per mm^3.

In the formula: X = number of mast cells per mm^3
n = number of counted cells (nuclei) per mm^2 of examined tissue
a = thickness of cut
d = diameter of nucleus
h = diameter of nuclear segment discernible in microscope.

Results.

The result of the counting of the cells is given in Table II. The capsula hepatis of cattle shows the largest number of mast cells and the number of these cells is the multiple of the number found in other examined tissues.

The next in order, with respect to a high content of mast cells, is pleura pulmonalis, but the number here is only a small fraction of

Table II.

	Cattle	
	Mast cells per mm²	Mast cells per mm³
Pleura parietalis	65.3 ± 5.8	5 022 ± 446
Pleura pulmonalis	126.0 ± 14.4	9,689 ± 1,107
Pericardium	43.2 ± 5.7	3.322 ± 438
Capsula hepatis	534.5 ± 54.0	41,103 ± 4,135
	Horse	
	Mast cells per mm²	Mast cells per mm³
Pleura parietalis	14.9 ± 5.0	1,146 ± 384
Pleura pulmonalis	65.0 ± 7.1	4,998 ± 546
Pericardium	10.6 ± 1.7	815 ± 131
Capsula hepatis	55.8 ± 7.6	4,291 ± 584

the number in capsula hepatis. The other membranes examined showed a lower number of mast cells.

The number of mast cells in the corresponding membranes of the horse is lower than that in the corresponding parts in cattle. In capsula hepatis it was of the same order of magnitude as in pleura pulmonalis. In pleura parietalis and in pericardium the number was throughout lower.

Discussion.

The above assay shows, as had been found before by other authors, that the number of mast cells in capsula hepatis of cattle is extremely high. In pleura pulmonalis it is only one fifth of the number in capsula hepatis. This is largely in accordance with the content of heparin in these tissues (WILANDER 1938), but it is not in accordance with recent histamine assays, the histamine content of the ox pleura, being found to be very high (RILEY and WEST 1953). The question arises how to explain these findings.

It is known that the content of histamine varies greatly in the different tissues of the same animal, and great individual variations are said to occur (SMITH 1953). When assaying histamine in different tissues of the cat, SMITH found that the greatest quantity of histamine was found in the ear hide (95 μg/g), followed by the duodenum (40 μg/g) and the lung (35 μg/g). The histamine content of the liver

(2.8 μg/g) was only ten percent of the content of the lung. Possibly these findings may have some bearing on those made in cattle. The most probable explanation, however, is that histamine can be liberated not only in the mast cells, but also from other elements of the tissues.

Summary.

The number of mast cells per area and volume have been counted in the pleura, pericardium and capsula hepatis of cattle and horse.

The capsula hepatis of cattle contains a 5—10 times larger number of mast cells than the other tissues. The pleura pulmonalis showed a content of mast cells which was only a fraction of that in the liver capsule. The high histamine content found by RILEY and coworkers when analyzing ox pleura seem to indicate that histamine is liberated not only from the mast cells, but also from other elements of the tissues.

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The Effect of Yohimbine and Adrenaline on the Oxygen Consumption.

By

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Received 22 October 1955.

MOHME and LUNDHOLM (1949) found that in guinea-pigs ergotamine inhibited the stimulating effect of adrenaline on the oxygen consumption. LUNDHOLM (1949 a) later demonstrated the mechanism of action: ergotamine blocked the lactic acid forming effect of adrenaline, and the latter's stimulating effect on the oxygen consumption was associated with oxidation of the lactic acid formed.

It seemed worth while to find out whether yet another classic sympathicolytic — yohimbine — also inhibited the calorigenic effect of adrenaline by blocking its lactic acid forming action. WEGER (1935) had shown, in rats, that yohimbine in a dose of 1—18 mg per kg body weight inhibited the stimulating effect of adrenaline, in a dose of 1—2 mg per kg, on the oxygen consumption. In experiments on guinea-pigs I first verified that yohimbine inhibited the calorigenic effect of adrenaline; and in further experiments on rabbits it emerged that this depression of the oxygen consumption was due to the fact that yohimbine also inhibited the lactic acid forming effect of adrenaline.

I. Experiments on Guinea-Pigs.

Methods.

The oxygen consumption in guinea-pigs was determined with the use of an apparatus previously described by LUNDHOLM and MOHME (1949). In order to reduce experimental variations, experiments were conducted simultaneously on four animals enclosed in *one* respiration chamber. The oxygen consumption values in the tables accordingly refer to the total oxygen consumption of the four animals in each group. The advantages and drawbacks of the method have been discussed by LUNDHOLM (1949 b).

The groups of guinea-pigs were composed of animals of the same sex and approximately the same weight, the individual weights ranging between 440 and 920 gm. The temperature in the respiration chamber was + 25° C.

The experimental procedure was as follows. The animals were deprived of food for 15 hours, then given a subcutaneous injection of 1 ml normal saline. The basal oxygen consumption was then recorded for 60 minutes, after which the injections of the various drugs were given. Adrenaline was administered subcutaneously in a dose of 20 μg per kg. Recording of the oxygen consumption commenced 5 minutes after the injection and covered five-minute periods. Yohimbine was given intramuscularly in a dose of 1 mg per kg, and recording of the oxygen consumption started 20 minutes after the injection. In the yohimbine-adrenaline experiments, 1 mg yohimbine per kg was first given intramuscularly, then 20 μg adrenaline per kg subcutaneously after 15 minutes. Recording of the oxygen consumption began 5 minutes after the injection of adrenaline and covered five-minute periods.

These experiments were conducted on four groups each comprising four guinea-pigs. Each group was subjected to two experiments with adrenaline, two with yohimbine, and two with yohimbine-adrenaline.

Results.

These are shown in Table I and figure 1. From the latter it will be seen that both adrenaline and yohimbine had a stimulating effect on the oxygen consumption. This latter increased in the adrenaline experiments by a maximum of 18 per cent and in the yohimbine experiments by a maximum of 18 per cent of the basal value. In the yohimbine-adrenaline experiments the oxygen consumption initially rose by 29 per cent, but after only 10 minutes the curve coincided with that in the yohimbine experiments. Figure 1 also shows the oxygen consumption curve that would have resulted had the effects of adrenaline and yohimbine been added to each other, on the assumption that yohimbine did not block the action of adrenaline. The curves are quite different, aside from the first 10 minutes.

Table I.

The effect of adrenaline, yohimbine and yohimbine-adrenaline on the oxygen consumption of the guinea pig.

Group and weight of animals		Yohimbine 1 mg/kg		Adrenaline 20 μg/kg		Yohimbine-adrenaline	
No.	g	Basal value O_2 ml/60 min.	Increase in per cent of basal value 20—115 min. after the injection	Basal value O_2 ml/60 min.	Increase in per cent of basal value 5—100 min. after the injection	Basal value O_2 ml/60 min.	Increase in per cent of basal value 5—100 min. after the injection of adrenaline
1	750	1931	24.9	1668	24.1	1996	18.4
	715						
	690	1892	17.3	1782	14.9	2062	29.6
	525						
2	630	1916	11.2	1683	21.0	1695	12.1
	575						
	560	1640	16.3	1755	14.1	1764	8.6
	450						
3	830	2042	5.0	1539	8.3	2196	11.7
	750						
	650	2120	5.4	1473	15.3	2048	14.3
	630						
4	920	1922	15.7	1828	11.4	2019	16.8
	830						
	780	2098	18.1	2357	12.6	2044	3.2
	770						
Mean			14.2		15.2		14.3
			$P < 0.001$		$P < 0.001$		$0.01 > P > 0.001$

Difference: Adrenaline + Yohimbine − Yohimbine-adrenaline = 15.1 ±4.1
$0.01 > P > 0.001$

In Table I is shown both the basal oxygen consumption over a 60-minute period and the increase, in relation to the basal value, 5—100 minutes after injection of adrenaline in the adrenaline and the yohimbine-adrenaline experiments. In the yohimbine experiments the increase is shown 20—115 minutes after the injection. The rise of oxygen consumption was in each case statistically significant, as will be seen from the table. If yohimbine did not inhibit the effect of adrenaline on the oxygen consumption, then the sum of the separate effects of the two drugs should be identical with the combined action

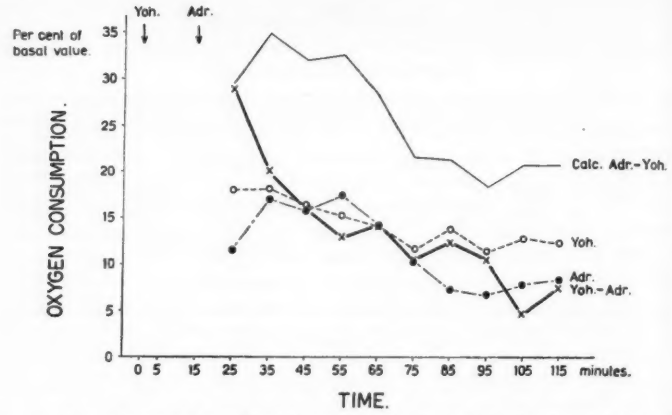

Fig. 1. The effect of adrenaline (20 μg per kg), yohimbine (1 mg per kg), and yohimbine-adrenaline on the oxygen consumption of the guinea-pig. Each point is the mean of eight determinations.

of yohimbine-adrenaline. It is clear from the table, however, that a difference existed between these values and that it was statistically highly probable; *i.e.*, yohimbine seems to have blocked the effect of adrenaline.

II. Experiments on Rabbits.

Methods.

The experimental procedure was as follows. After the animals had been deprived of food for 15–18 hours, two fine cannulae were inserted under local anesthesia, one in the central artery of the ear and the other in a marginal vein of the ear. The cannulae were attached by means of adhesive tape. The rabbit then received 1 ml of a 5 per cent heparin solution intravenously. After preparation the animal was placed in the respiration chamber of the apparatus described by LUNDHOLM (1949 b) for recording of the oxygen consumption. The temperature therein was + 24° C. From the animal thus enclosed, arterial blood could be taken for lactic acid assay, and drugs injected or infused intravenously, via fine rubber tubes running from the cannulae.

When the oxygen consumption had become stabilized, it was recorded for a period of 60 minutes. In the adrenaline experiments, 0.3 μg per kg per minute was then infused intravenously for 60 minutes with the use of an infusion apparatus. The adrenaline was dissolved in normal saline which had been deoxidized by boiling, to prevent oxidation of the adrenaline.

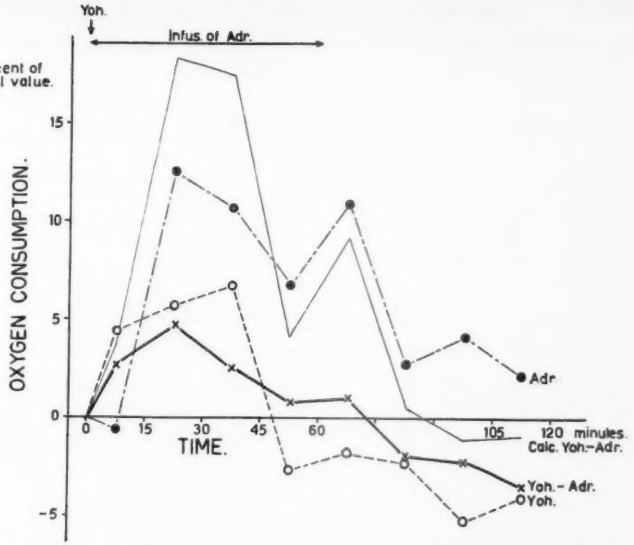

Fig. 2. The effect on the oxygen consumption, in the rabbit, of yohimbine (1 mg per kg) in intravenous injection, adrenaline in intravenous infusion for 60 minutes in a dose of 0.3 μg per kg per minute, and yohimbine-adrenaline. Each point is the mean of five to six determinations.

The rate of infusion was 0.3 ml per minute. Yohimbine hydrochloride was given intravenously in a dose of 1 mg per kg. In the yohimbine-adrenaline experiments the former drug was given immediately, before the infusion of adrenaline began.

Blood samples for lactic acid assay were taken, in all experiments, immediately before any drug was given; thereafter they were taken, in the yohimbine series, 10, 20, 30, 40, 60 and 90 minutes after the injection, and in the adrenaline and yohimbine-adrenaline series 30, 60, 90 and 120 minutes after the start of infusion. The lactic acid content was determined by the method of FRIEDEMANN, SHAFFER and COTONIO (1933).

Results.

These are presented in figures 2 and 3 and tables II and III. In the adrenaline series the oxygen consumption increased by a maximum of 12.5 per cent. The average increase, up to 120 minutes after the start of infusion, was 6.0 per cent. Yohimbine, too, initially had a slight stimulating effect on the oxygen consumption, which rose by a

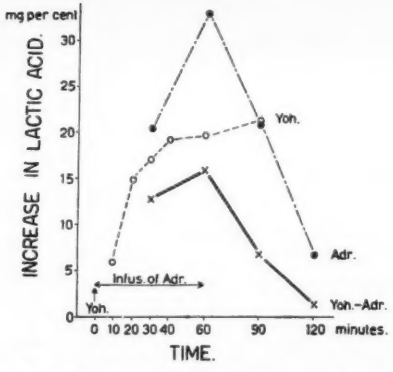

Fig. 3. The effect of yohimbine, adrenaline, and yohimbine-adrenaline on the lactic acid content of the blood in the rabbit. Each point is the mean of five to six determinations.

maximum of 6.5 per cent. Yet after about 50 minutes it fell below the basal value and declined to a minimum of — 5.0 per cent. However, neither the initial elevation nor the later depression could be statistically verified. The average increase in oxygen consumption up to 120 minutes after the injection of yohimbine was 0.4 per cent. In the yohimbine-adrenaline series the oxygen consumption curve coincided very closely with that in the yohimbine experiments, and no stimulating effect of adrenaline was demonstrable. From Table II it will be seen that the difference between the sum of the separate effects of yohimbine and adrenaline minus the effect of yohimbine-adrenaline was statistically probable; *i.e.*, yohimbine had blocked the stimulating effect of adrenaline on the oxygen consumption.

The effects of yohimbine and adrenaline on the lactic acid content of the blood are presented in figure 3, showing the increase above the basal value. The basal lactic acid content averaged 8.1 mg per cent in both the yohimbine and the adrenaline series, and 17.0 mg per cent in the yohimbine-adrenaline series.

Both adrenaline and yohimbine, as shown in figure 3, raised the lactic acid content of the blood. Yohimbine increased it by an average of 20 mg per cent 40—90 minutes after the injection, whereas adrenaline at the end of the infusion had increased it by 33 mg per cent, on

Table II.

The effect of adrenaline, yohimbine and yohimbine-adrenaline on the oxygen consumption of the rabbit.

Yohimbine 1 mg/kg			Adrenaline 0.3 μg/kg/min. for 60 minutes			Yohimbine-adrenaline		
Weight kg	Basal value O_2 ml/ 60 min.	Deviation in per cent of basal value 0—120 min. after the injection	Weight kg	Basal value O_2 ml/ 60 min.	Deviation in per cent of basal value 0—120 min. after start of the infusion	Weight kg	Basal value O_2 ml/ 60 min.	Deviation in per cent of basal value 0—120 min. after the injections
4.35	1829	+ 1.7	2.90	1541	+ 9.6	1.85	934	− 0.9
2.90	1366	+ 1.4	2.40	1336	+ 1.5	2.80	1560	− 5.7
3.00	1953	+ 1.7	2.70	1505	+ 6.7	2.20	1318	+ 3.5
2.55	1860	− 1.8	2.40	1380	+ 1.2	2.40	1420	− 1.8
2.60	1502	− 0.9	2.45	1262	+11.8	2.25	1387	+ 3.8
			2.40	1423	+ 5.2			
Mean		+ 0.41			+ 6.0			+ 0.14
		$0.5 > P > 0.4$			$0.02 > P > 0.01$			$P > 0.9$

Difference: Adrenaline + Yohimbine − Yohimbine-adrenaline = 6.3 ± 2.58
$0.05 > P > 0.02$

the average. However, the curve relating to yohimbine and adrenaline in combination was lower than both the yohimbine and the adrenaline curves.

Table III shows the values for the lactic acid content of the blood of different rabbits 60 minutes after the injection of yohimbine and immediately after the end of the infusion of adrenaline. In each case the increase could be statistically verified, as could also the inhibition by yohimbine of the lactic acid forming effect of adrenaline.

Discussion.

The effect of yohimbine on the oxygen consumption seemed to vary in different species of animals. In guinea-pigs it was stimulative, but in rabbits no definite effect was demonstrable. MURAKAMI (1930), however, reported a slight depressive effect in the latter animal. WEGER (1935), who studied the oxygen consumption in the rat, was

Table III.

The effect of adrenaline, yohimbine and yohimbine-adrenaline on the lactic acid content of the blood 60 minutes after the beginning of the injections. Increases over the basal values.

Yohimbine tests Mg per cent	Adrenaline tests Mg per cent	Yohimbine-adrenaline tests Mg per cent
9.0	43.2	9.3
21.0	42.6	25.2
13.3	14.8	16.1
27.2	22.6	22.2
26.9	38.0	6.5
	37.0	
Mean 19.5	33.0	15.8
$0.01 > P > 0.001$	$P < 0.001$	$0.02 > P > 0.01$

Difference: Adrenaline + Yohimbine − Yohimbine-adrenaline = 36.7 ± 7.0
$P < 0.001$

unable to demonstrate any effect of yohimbine in doses between 1 mg and 18 mg per kg.

Irrespective of the action of yohimbine itself on the metabolism, it completely blocked the stimulating effect of adrenaline on the oxygen consumption by inhibiting the lactic acid forming action of that substance. Moreover, yohimbine itself elevated the lactic acid content of the blood. This was probably a specific effect; not a non-specific, toxic one. In guinea-pigs, for instance, the lethal dose of yohimbine, when injected intraperitoneally, is about 40 mg per kg (RAYMOND-HAMET), *i.e.*, forty times the dose used by me. Yohimbine also has a stimulating action on the blood sugar, at the same time as it inhibits the hyperglycemic effect of adrenaline (NITZESKU 1928, HANSSON 1929). Since the hyperglycemic effect of yohimbine persists after transection of the splanchnic nerve, it is probably of peripheral nature (IMAHASI 1928).

The fact that both yohimbine and adrenaline elevate the blood sugar and blood lactic acid, and that yohimbine may block these effects of adrenaline, suggests that the two drugs have an affinity for the same receptor mechanism and that yohimbine may block the effect of adrenaline by competitive inhibition.

Summary.

The effect of yohimbine, adrenaline, and a combination of the two on the oxygen consumption and the lactic acid content of the blood was studied in guinea-pigs and rabbits.

In guinea-pigs, yohimbine in an intramuscular injection of 1 mg per kg increased the oxygen consumption by an average of 14.2 per cent between 20 and 115 minutes after the injection.

In rabbits, yohimbine in an intravenous injection of 1 mg per kg had no demonstrable effect on the oxygen consumption.

In both guinea-pigs and rabbits, yohimbine totally inhibited the stimulating effect of adrenaline on the oxygen consumption.

In the rabbit experiments the effects of yohimbine and adrenaline on the lactic acid content of the blood were also investigated. Each of those drugs produced an elevation. When yohimbine and adrenaline were administered jointly, the former totally inhibited the lactic acid forming effect of the latter.

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The Alkaline Taste.

By

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It has been emphasized that an alkaline solution in the mouth may stimulate different kinds of receptors, such as those of smell, pressure, pain and temperature as well as those of taste (VON SKRAMLIK 1926). Though the complex nature of the alkaline taste seems to be generally accepted, it still remains undecided whether there is also a stimulation of specific taste receptors. According to VON FREY (1910) alkaline taste does not, as a rule, appear until the fluid has been swallowed. He observed that the introduction of 10 ml 0.01 M sodium hydroxide into the mouth of a subject with a clip on his nose evoked from the tip of the tongue a sweet sensation, and from the base of the tongue a slightly bitter sensation; there was also a suggestion of burning and, if the tongue were moved about, a feeling of smoothness. An alkaline taste proper did not become evident until the clip had been removed. VON FREY therefore supposed that it was due to smell and caused by volatile bases (methylated ammonia) from decomposed epithelial cells. HAHN, KUCKULIES and TAEGER (1938), however, irrigating a small area of the tongue through a hole at the bottom of a U-shaped glass tube, still observed a specific alkaline taste. It had a threshold

value of 33.10^{-5} M — $13.5.10^{-5}$ M, whereas the corresponding values for the sweet taste of the alkaline solutions were 100—1000 times higher.

Using the method of correct and false cases as described in an earlier publication (LILJESTRAND and ZOTTERMAN 1954) one of us (G. L.) made some determinations on himself of the threshold value for sodium hydroxide in tap water. Between the samples the mouth was rinsed with very dilute hydrochloric acid and then with tap water. It turned out that it was possible to differentiate between 0.0025 M sodium hydroxide (pH 11.1) and tap water (pH 7.8) with 100 per cent certainty, and practically the same result was obtained if the nose of the subject had been clamped with a clip during the experiments. With borate buffer solutions containing the same percentage of borate, it was possible to differentiate between pH 9.8 and 9.2 (control). These results indicate a sensitivity of the taste organs for a deviation of the pH on the alkaline side about as great as that which is known to exist on the acid side, the threshold value for hydrochloric acid under corresponding conditions being about pH 3.5 (LILJESTRAND 1922).

MONCRIEFF (1948) has considered the alkaline taste to be a function not of taste but of the "common chemical sense", since the alkalies are chemically irritating to other parts of the body as well as the tongue. He has pointed out that a comparison between the sensitivity of the tip of the tongue and that of the dorsum of the tongue might enable a differentiation on this point. Such a comparison has been performed by KLOEHN and BROGDEN (1948) who found a much lower threshold value for alkali at the tip than at the dorsum linguae. They conclude that the evidence, such as it is, lies in favour of the hypothesis that the gustatory receptors are involved in the taste of alkalinity, though it is a complex and not a primary taste quality.

The following study was undertaken in the hope that it might be possible by the aid of the action potentials from the chorda tympani nerve to obtain further information with regard to the mode of action behind the alkaline taste.

Technique.

In all 21 cats were used. The animals were anaestetized by intramuscular injection of a chloralose-urethane solution (0.50 g chloralose and 0.25 g urethane in 7 ml Ringer's solution per kg body weight). The right chorda

tympani was prepared; the whole nerve or few fibre preparations were used as described in earlier papers (LILJESTRAND and ZOTTERMAN 1954, COHEN, HAGIWARA and ZOTTERMAN 1955), the action potentials were led off and the test solutions applied in the way described before. The threshold concentration of alkaline solutions was determined also by leading off from the whole chorda tympani nerve using an integrating device which allowed a quantitative estimation of the total nerve activity. The temperature of the solutions was kept at 26°−28° C. The following solutions were used as stimulants: distilled water, 0.5 M sodium chloride, 0.01 M quinine chloride in 0.1 M sodium chloride, hydrochloric acid in 0.1 M sodium chloride (or Ringer's solution) at pH 2.5−2.9, sodium hydroxide in 0.1 M sodium chloride (or Ringer's solution) at pH 11.0−12.3. We sometimes found it convenient to prepare the alkaline solutions by adding 0.1 M sodium hydroxide to 0.1 M sodium chloride until the solution evolved a definite alkaline but not unpleasant taste when applied on the human tongue. After the experiments the pH was determined with a Beckman pH meter. The values given have not been corrected for the salt error. In a few cases we also used borate buffer solutions (M boric acid with varying amounts of sodium hydroxide).

Results.

Whole Nerve.

When action potentials were led off from the whole chorda tympani nerve, the application of a solution of sufficient alkalinity to the tongue was regularly followed by a continuous discharge of impulses. If the alkaline solution was made up of sodium hydroxide in 0.1 M sodium chloride (or Ringer's solution) usually no effect was observed at a pH of 11.0 or less, but as the alkalinity increased the response became obvious, and at about pH 12 it was generally quite pronounced (Fig. 1). The general appearance of the potentials was the same as that obtained after application of water, 0.5 M sodium chloride, 0.01 M quinine chloride or acid (pH 2.5). If a buffered borate solution was used, no effect occurred at pH 7.5, but a moderate response was obtained at about pH 9 and a stronger response at pH 10.7.

The application of a cotton pad, thouroughly soaked with a solution of 0.1 M sodium chloride with an addition of sodium hydroxide to pH 12.4 on the dorsum linguae was followed by a moderate positive response. From the tip of the tongue the same solution led to a more pronounced reply.

The threshold value for alkaline solutions was ascertained with the integrating device mentioned above. Fig. 1 gives examples of tracings

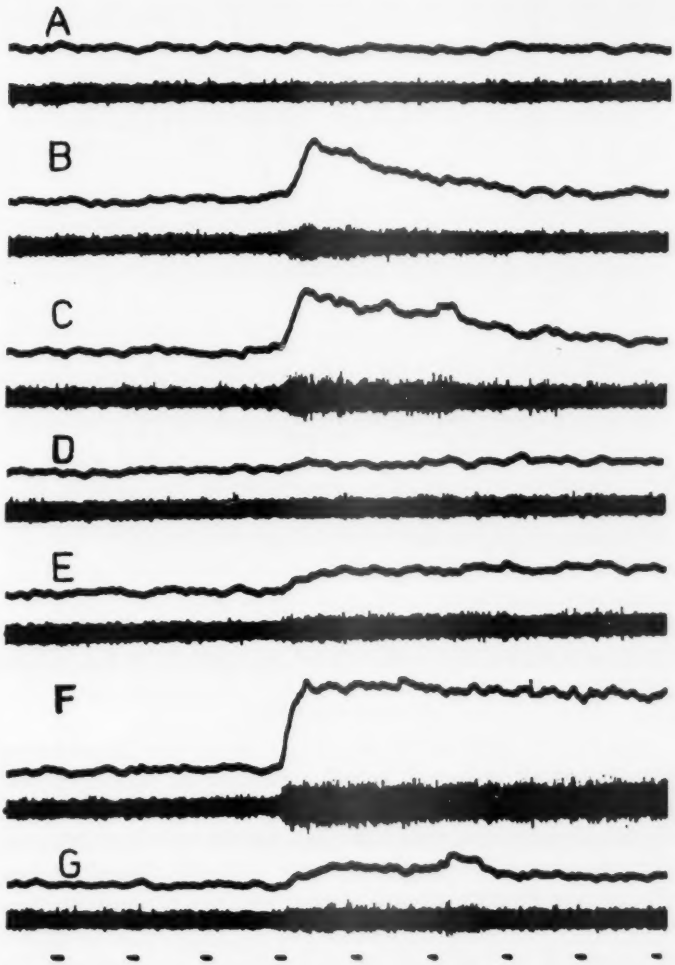

Fig. 1. Records from the whole chorda tympani nerve of cat when applying to the tongue *A*. Ringer at 28° C. *B*. Ringer at 35° C. *C*. Distilled water. *D*, *E* and *F*. Ringer's solution with sodium hydroxide added to pH 11.7 (*D*), 11.9 (*E*) and 12.2 (*F*). *G*. Distilled water 5 minutes later.
Upper tracing integrated response. Time in seconds. All test solutions but in *B* at 28° C.

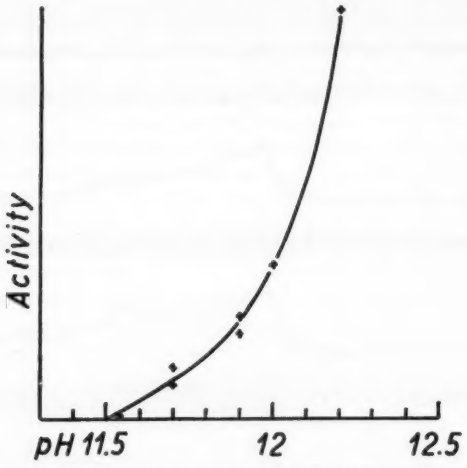

Fig. 2. Quantitative relations between integrated activity from the whole chorda tympani nerve of cat and Ringer's solution at different alkalinities.

obtained in this way. A stimulating effect starts a little above pH 11.5; it then rises rapidly with increasing pH, as illustrated in the diagram (Fig. 2).

Few Fibre preparations.

As a rule our preparations with only a few chorda tympani nerve fibres responded to warmth as well as to chemical stimuli, but this was not always the case. Thus we have had several preparations which did not display any increased electrical activity after the application of warm or even hot Ringer's solution (37°—46° C) and once no effect of cold Ringer's, whereas alkaline solutions produced excellent responses. We have often observed that the response to touch was absent though that to alkali was still present. The reverse situation may sometimes prevail. Thus we have twice seen typical positive effects from touch but no response to alkali or other chemical stimuli. In few fibre preparations from the lingual nerve we have never observed increase in the action potentials after stimulating the tongue with alkaline solutions, though warmth or cold, touch or painful stimuli evoked strong typical responses. It has thus been possible to dissociate the stimulating action of alkali from that of touch or cold

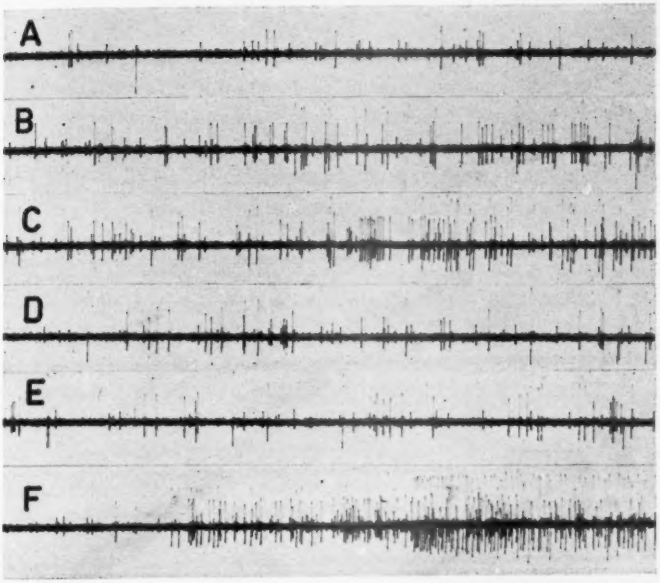

Fig. 3. Records from chorda tympani "water fibres" of cat when applying to the tongue *A*. Ringer's solution. *B*. Distilled water. *C*. 0.9 per cent sodium chloride with sodium hydroxide added to pH 12.1. *D*. 0.5 M sodium chloride. *E*. 0.01 M quinine chloride in Ringer's solution. *F*. Hydrochloric acid in 0.9 per cent sodium chloride, pH 2.9. Speed of record as in Fig. 4.

or warmth (or pain). We conclude that alkali must exercise an effect on the taste receptors just as other chemoceptive substances do.

"Water Fibres".

In 16 preparations from 10 cats we obtained fibres which responded to water but not to salt. In some of these cases quinine also provoked an increased electrical activity, but in at least 6 cases such an effect was absent. With one doubtful exception the water fibres always responded to alkali. Solutions of pH above 12.1 depressed the subsequent response to water to very much the same extent as strong acids do (Cp COHEN, HAGIWARA and ZOTTERMAN). Fig. 3 illustrates some of the responses just described.

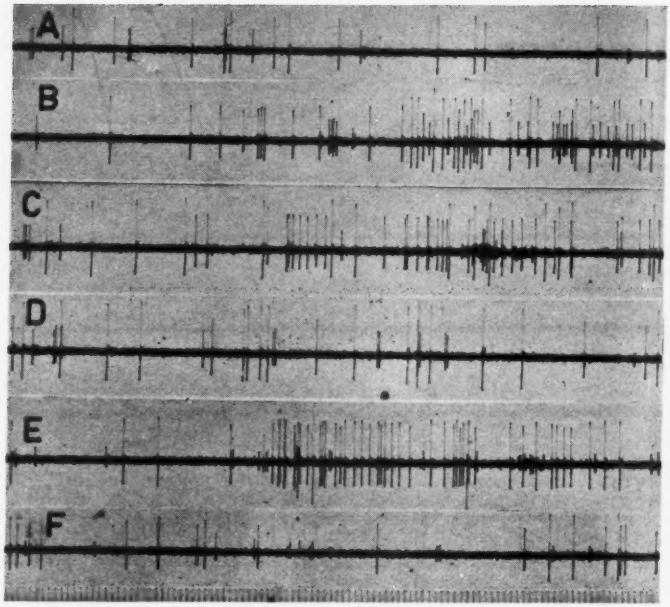

Fig. 4. Records from chorda tympani "salt fibres" of cat when applying to the tongue *A*. Ringer's solution. *B*. 0.5 M sodium chloride. *C*. 0.9 per cent sodium chloride with sodium hydroxide added to pH 12.1. *D*. Distilled water. *E*. Hydrochloric acid in 0.9 per cent sodium chloride, pH 2.9. *F*. 0.01 M quinine chloride in 0.9 per cent sodium chloride. Time marker 50 cycles per second.

Salt Fibres.

Twelve preparations from 8 cats responded to 0.5 M sodium chloride but not to water. In about half of them quinine also elicited a positive response. In all cases, with but one exception, the application of alkali was followed by an increased electrical activity. Fig. 4 illustrates a typical experiment of this kind, in which quinine had no effect.

"Quinine Fibres".

In 3 cats 5 preparations were obtained which responded to 0.01 M quinine chloride but neither to water nor to 0.5 M sodium chloride. One of them was tested with acid with a negative result. This preparation as well as two of the others responded to alkali, whereas in a fourth preparation alkali had no effect.

Discussion.

The electrical responses from the chorda tympani nerve obtained in this study after the application of alkaline solutions to the tongue cannot be the result of a stimulation of receptors for touch, cold or warmth (or pain), since the same results have been obtained when the last-mentioned stimuli were without effect. There can be no doubt that under the conditions of our experiments alkali has had a pronounced stimulating influence on taste receptors. This conclusion is in agreement with the findings of KLOEHN and BROGDEN quoted above. Our own observations also indicate less sensitivity for alkali on the dorsum linguae than on the tip of the tongue. It is possible that higher degrees of alkalinity than those used in this investigation may stimulate all kinds of receptors (even touch) just as may be the case with strong acids, but direct evidence on this point is not available at present. If the alkaline taste is regarded as a function of a "common chemical sense", this also would be true for the sour taste; in both cases, however, the taste receptors are evidently specially adapted to a much greater sensitivity for these stimuli than other receptors.

There is on the whole a rather close analogy between alkalies and acids with regard to their general action on the taste receptors. The deviation from the neutral point necessary to exert a non-specific stimulation of nearly all kinds of taste fibres is about the same in either direction, as mentioned previously.

Whereas only special fibres are sensitive for water, salt or quinine, practically all of them respond to acids as well as to alkali. We have mentioned two exceptions with regard to the stimulating effect of alkali which was not observed in one water fibre and one salt fibre. None of these two preparations reacted very strongly, and no records were taken. The same situation was once observed with one "specific" acid fibre which responded to acid but neither to water, salt, quinine nor alkali. We are therefore inclined to consider the results as somewhat uncertain. The negative result obtained after alkali when a record was taken from a quinine fibre corresponds well to the observations of COHEN, HAGIWARA and ZOTTERMAN that quinine fibres are not stimulated at all or very slightly by strong acids. This observation was once confirmed in this study, and it seems of special interest that in this particular case the fibre responded to alkali. A similarity between alkalies and acids is the depressing influence on the sub-

sequent stimulating action of water (Fig. 3). COHEN, HAGIWARA and ZOTTERMAN on two occasions observed functional single fibres which responded only to acids. We have never seen corresponding fibres responding only to alkali.

PFAFFMANN (1941) has pointed out that the acid taste probably results from a general stimulation of all taste fibre endings. COHEN, HAGIWARA and ZOTTERMAN have confirmed his findings that strong acids stimulate the endings of "salt" and "water" fibres and to a minor degree only the "quinine" fibres in the cat. In addition they found specific acid fibres the endings of which did not respond to anything but acid and which were sensitive to acids of less acidity than that necessary for stimulating other taste fibre endings. Since alkali has been found to stimulate water, salt and to some extent also quinine fibres, the situation is very similar to the non-specific response to acids. It must be assumed, however, that there are considerable quantitative differences so that the relative part played by each kind of fibre endings varies. In this way the total input pattern will be quite different in the different cases. At present it is not possible to decide whether and to which extent such is the case.

Strong acids produce a change of the physical properties of the mucous membranes giving rise to an adstringent sensation which is mediated by mechanoceptors of the tongue. Similarly alkali produces the opposite effect of a feeling of a softer surface, which may play its rôle in the discrimination. Thus acid and alkaline solution can be discriminated not only by differences in the pattern of taste fibre impulses but as well by different pattern of mechanoceptive impulses.

It is interesting to note in this connection that alkali may give rise to a very pronounced sweet taste, *e.g.* when the mouth is washed with water after the application of a 0.1 M solution of sodium chloride with a pH about 12 — and also that a slightly bitter sensation after alkali has been observed as mentioned by VON FREY. It would seem natural to assume that these tastes are the result of a stimulation of the corresponding receptors by alkali under conditions where for some reason other receptors are less influenced. In this way the input pattern might well vary with the degree of alkalinity and other factors.

Summary.

The application of alkaline solutions to the tongue of the cats is followed by a continuous outburst of impulses in the chorda tympani nerve. The alkaline taste is the result of a general stimulation of several kinds of taste fibre endings. Experiments on few fibre preparations have shown that "water fibres", "salt fibres" and to some extent also "quinine fibres" are stimulated by alkali.

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